



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants: Samy Ashkar and Jairo Salcedo

Serial No.: 09/981,845

Art Unit: 1647

Filed: October 18, 2001

Examiner: Regina M. Deberry

For: *OSTEOPONTIN-COATED SURFACES AND METHODS OF USE*

Mail Stop Appeal Brief-Patents  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**APPEAL BRIEF**

Sir:

This is an appeal from the final rejection of claims 1-3, 5 and 6 in the Office Action mailed August 8, 2005, in the above-identified patent application. A Notice of Appeal was filed on November 8, 2005. An Advisory Action was mailed February 15, 2006. **An Appeal brief was filed on August 16, 2004, and a Substitute Appeal Brief on December 2, 2004. The examiner reopened examination on March 17, 2005, in response to the Substitute Appeal Brief. The Commissioner was authorized to charge \$165 to Appellant's deposit account in payment of the Appeal Brief filed on August 16, 2004, the fee for filing an Appeal Brief for a small entity. However, the fee for filing an Appeal Brief for a small entity is currently \$250.00. The Commissioner is hereby authorized to charge the difference of \$85.00, to Deposit Account No. 50-3129. Submitted with this Appeal Brief is a Petition for an Extension of Time, to extend the period for response for two months, to and including March 8, 2006. The**

Commissioner is hereby authorized to charge \$225.00, the fee for a small entity, to Deposit Account No. 50-3129. It is believed that no additional fee is required with this submission. However, should an additional fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

**(1) REAL PARTY IN INTEREST**

The real parties in interest of this application are Children's Medical Center Corporation in Boston, MA, the assignee of record.

**(2) RELATED APPEALS AND INTERFERENCES**

There are no related appeals or interferences known to the appellant, the undersigned, or appellant's assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

**(3) STATUS OF CLAIMS ON APPEAL**

Claims 1-3, 5 and 6 are pending. Claims 4 and 7-18 have been cancelled. Claims 1-3, 5 and 6 are on appeal. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

**(4) STATUS OF AMENDMENTS**

An amendment was filed on January 9, 2006, subsequent to the final rejection mailed August 8, 2005. In the advisory action mailed on February 15, 2006, the Examiner indicated that this amendment would be entered. Claims 4 was cancelled in the Amendment filed on June 15, 2005. Claims 7-18 were cancelled in an Amendment filed on November 21, 2003.

**(5) SUMMARY OF THE CLAIMED SUBJECT MATTER**

Independent claim 1 defines an active osteopontin peptide fragment comprising an amino acid sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID

NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein the peptide binds to at least one integrin receptor on a cell surface selected from the group consisting of  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $4 \beta 1$ ,  $2 \beta 1$ , VCAM, ICAM CD44, V3Vx (see at least page 8, lines 7-26 and page 12, lines 4-13).

Dependent claim 2 defines the active peptide fragment of claim 1, wherein the peptide increases cell attachment to a material and increases cell spread (see at least page 8, lines 11-12 and page 53, lines 12-17).

Dependent claim 3 defines the active peptide fragment of claim 1, wherein the peptide binds to at least one integrin receptor on a cell surface selected from the group consisting of VCAM, ICAM CD44, and V3Vx.

Dependent claim 5 defines the active peptide fragment of claim 1, wherein the integrin(s) is selected from the group consisting of  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $4 \beta 1$ , and  $2 \beta 1$ . Support for claims 3 and 5 can be found at least on page 3, line 27 to page 4, line 14 and page 53, lines 17-21.

Dependent claim 6 defines the active peptide fragment of claim 1, wherein the cell is an osteoprogenitor cell, tumor cell, macrophage, periosteal cell, endothelial cell, epithelial cell, eosinophil, stem cell, limited potential precursor cell, precursor cells committed precursor cell, or differentiated cell (see at least page 8, line 29 to page 9, line 2).

**(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

The issue presented on appeal is whether claims 1-3, 5 and 6 are enabled under 35 U.S.C. § 112, first paragraph.

**(7) GROUPING OF CLAIMS**

The claims do not stand or fall together. Arguments for the separate patentability of the claims are provided below.

## **(8) ARGUMENTS**

### **(a) The Claimed Invention**

Osseointegration is a complex process that involves proliferation, migration, attachment, differentiation, extracellular matrix synthesis, and finally mineralization of that matrix. Differentiated cells originate from “primitive” cells called stem cells, which are pluripotent and divide to generate committed precursor cells. After a series of rapid cell divisions, these committed precursor cells develop into differentiated cells, wherein a contribution is made to the surrounding matrix. Driving this process of cellular development is motility and proliferation, which are in turn regulated by increasing or decreasing gradients of, for example, peptides which bind to receptors on the cell surface. The bone trauma generated by implant placement is followed clot formation, acute inflammation, recruitment and proliferation of stromal cells and their differentiation into osteogenic lineage cells, followed by filling the defect with bone and finally mineralization of the matrix.

Extracellular matrix proteins, especially adhesion molecules, play a role in bone repair and morphogenesis. When cells initially encounter a bio-matrix or extracellular matrix (“ECM”), they will either attach and spread or undergo apoptosis. Adherence to the ECM is a receptor-mediated process. Cell surface receptors belonging to the integrin superfamily are recognized as critical players in the adhesion to the ECM and are intermediate messengers relaying signals for events such as contact, anchorage, and differentiation. Proteins such as osteopontin or peptides derived from osteopontin, which bind to these receptors can therefore mediate these cellular processes.

The primary challenge faced in the fabrication of new implants is to increase the rate of osseointegration and the percentage of bone apposition. An enhanced rate of osseointegration and/or augmented percentage of bone apposition around implants increases implant placement indications, and expedites loading time. Recent studies have focused on improving osseointegration of implants by coating the surface with various substances including bone morphological proteins, with varying degrees of success.

The Appellants have isolated active osteopontin peptide fragments that have cell-attachment and cell-spread activity, which, when coated on a material suitable for use as an implant, can increase cell attachment as well as enhance cell spread. The peptides discovered by the Appellant help bring stem cells, precursor cells and differentiated cells into contact with the material. They can also function in bringing tissue remodeling cells such as mesenchymal, macrophages and granulocytes and in general cells that are involved in osseointegration, into contact with the implant.

**(b) Rejection of claims 1-3, 5 and 6 Under 35 U.S.C. § 112, first paragraph, enablement**

***The Legal Standard for Enablement***

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir.1988). As set forth in *Wands*, the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the

breadth of the claims. In cases that involve unpredictable factors, "the scope of the enablement obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation 'must not be unduly extensive.' *Atlas Powder Co., v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984). There is no requirement for examples. The Supreme Court also noted that all of the factors need not be reviewed when determining whether a disclosure is enabling *In re Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991) (noting that the *Wands* factors 'are illustrative, not mandatory. What is relevant depends on the facts.'). As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied. *In re Douglas v. United States* 510 F.2d 364; 184 U.S.P.Q. 613 (Ct. Cl.1975) the Court of Claims noted that a patentee cannot "be expected to foresee every technological problem that may be encountered in adapting his idea to a particular use. Some experimentation and exercise of judgment is to be expected. *In re Mineral Separation v. Hyde* 242 U.S. (1916), the court emphasized that some inventions cannot be practiced without adjustments being made to adapt them to the particular context. In such a situation, a specification is sufficient if it gives adequate guidance to one skilled in the art on how such adjustments are to be made.

### ***Analysis***

Claim 1 defines an active osteopontin peptide fragment comprising an amino acid sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID

NO:15, wherein the peptide binds to at least one integrin receptor on a cell surface selected from the group consisting of  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $4 \beta 1$ ,  $2 \beta 1$ , VCAM, ICAM CD44, V3Vx. The amino acid sequences of the peptides are disclosed (page 8, lines 7-26), and the specification on page 11, lines 9-11 and on page 12, lines 20-31 to page 13, lines 1-5, discloses how osteopontin can be modified to obtain the claimed peptides. The structure of osteopontin is well known in the art. It would therefore be routine for one skilled in the art to make the claimed peptides from the known structure of osteopontin, or, with knowledge of the disclosed sequences use other well known synthetic techniques.

An active osteopontin peptide refers to an osteopontin fragment that possesses at least one biological activity of naturally occurring osteopontin (see page 11, lines 9-11). The biological activity of osteopontin that the claimed peptides have includes cell attachment and cell spreading activity. Osteopontin performs these biological functions by binding to receptors on the cell surface. It is well known in the art that osteopontin binds to more than one integrin receptor, as is exemplified by Hu, et al, *J. Biol. Chem.* 270(11):26232-38 (1995) (see evidence appendix). The blast 2 sequence comparison (see evidence appendix) shows for example that SEQ ID NO 11 and 15 have conserved domains similar to osteopontin. One of ordinary skill in the art would expect the claimed peptides to bind to receptors capable of binding osteopontin. This ability of the peptides recited in claim 1 to bind to **at least one** integrin receptor on the cell surface is in fact demonstrated by the ability of anti-integrin antibodies to inhibit cell attachment (for example, SEQ ID NO: 15, (Table 8)). Although there is no need for examples, this example clearly demonstrates that the claimed peptides do indeed bind to integrins. There is no legal requirement that the claimed peptides bind all integrins or to all cell types for the peptides to have the specified utility. There is no legal requirement for actual reduction to practice. As long

as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied. Therefore claim 1, which recites that peptides consisting of SEQ ID Nos. 7-15, bind to at least one integrin receptor on a cell surface selected from the group of receptors recited in claim 1, claim 3 which recites that the peptide fragments of claim 1 bind to *at least one* integrin receptor selected from the integrin receptors listed in claim 3, and claim 5, which recites that the peptide fragments of claim 1 bind to at least on integrin on a cell surface, wherein the integrin is selected from the group consisting of the integrins listed in claim 5 are enabled.

The peptides may be used to increase cell attachment to a biomaterial and to increase cell spreading. The specification on page 13, line 14 to page 14, line 2 describes how to coat the peptides on a material, and the types of materials that may be coated (page 10, lines 16-23 and page 14, lines 22-28). The specification on page 40, lines 4-31, and page 41, lines 1-8, describes how to measure cell attachment and cell spreading. Example 12 shows the ability of the claimed peptides to increase cell attachment and cell spreading. Therefore, it is clear that claim 2, which recites osteopontin peptide fragments which bind to at least one integrin receptor of the surface of a cell and increases attachment to a material and increases cell spreading, is enabled.

Integrins are the principal receptors on animal cells for binding most extracellular matrix proteins, including collagen, fibronectin, and laminin. They are found on the surface of numerous cell types (see, for example, *Molecular Biology of the Cell*. IV. Cells in Their Social Context. 19. Cell Junctions, Cell Adhesion, and the Extracellular Matrix, Garland Publishing (1994)). As is exemplified in Hu, et al, *J. Biol. Chem.* 270(11):26232-38 (1995) and Tuck, et al, *J. Cell. Biochem.*, 78:465-75 (2000) (see evidence appendix), osteopontin would bind to



different cell types that express its receptors. The cells employed by Hu, et al. are carcinoma cells, whereas Tuck, et al. employed epithelial cells. These references demonstrate that osteopontin would bind to at least one of its receptors on a cell expressing the receptor. The claimed active osteopontin peptide fragments have the ability to bind integrins as already mentioned, and since integrins are expressed on diverse cell types, it would be expected that the claimed peptides would bind the diverse cell types expressing integrins. The specification describes a number of cell types that may be regulated using the active osteopontin peptides fragments (page 8, line 29 to page 9, line 2). Although there is no requirement for examples, Example 12 and Table 8 on pages 53-55, demonstrate that plates coated with each of, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15 bind to osteoprogenitor cells and significantly increases cellular attachment and spread over the control (uncoated plates). This is shown in table 8 with SEQ ID NO: 15, to be due to binding to the  $\alpha v \beta 3$  receptor. As emphasized previously by the examiner, osteoprogenitor cells were able to spread in the presence of antibodies to CD44 and  $\beta 1$ , showing that SEQ ID NO: 15 does not bind to these receptors in a manner that is supportive of osteoblast function. The only conclusion that can be drawn from table 8 is that cell attachment and spread is not predominantly controlled by CD44 and  $\beta 1$ . The study by Tuck et al., clearly supports this reasoning. Anti  $\alpha v \beta 5$  and  $\beta 1$  antibodies completely blocked migration of 21PT and 21NT cells but had no effect on migration of MDA-MB-435 cells (all mammary epithelial cells). Osteopontin-induced migration of MB-435 cells was completely blocked by anti-  $\alpha v \beta 3$  antibodies. This is not because osteopontin does not bind to these integrins (Hu, et al., states that it does), but because migration in these cell lines is dependent on different integrins. Furthermore, more malignant MDA-MB-435 cells express  $\alpha v \beta 3$  integrins, while less malignant cells do not (see Hu, et al. page 471).

Similar to this reasoning, Noonan, et al, J. Orthop. Res., 14(4):573-81 (1996) (abstract; see evidence appendix), states that osteoprogenitor cells express low levels of CD44. These references combined demonstrate that osteopontin or any peptide capable of binding to osteopontin receptors can perform the same biological function in different cell types depending on the receptor predominantly expressed by that cell. The claims are drawn to peptides that bind to at least one integrin receptor to increase cell binding and spread. As demonstrated above, for osteoprogenitor cells in particular, there should be very little expectation of a dramatic inhibition of cell spread, when an antibody to an integrin expressed at low levels is used. Table 8 demonstrated an 87% and 89% spread of osteoprogenitor cells in the presence of anti-CD 44 and anti- $\beta$ 1 antibodies, not a 100% spread. The fact that a peptide binds to at least one integrin receptor as stated in the claims to improve cell attachment and spread, does not exclude that fact that it could bind more than one, the amount of cell spread induced by a particular receptor being commensurate to the level of expression of that receptor. This is shown on table 8 and supported by Noonan, et al. A conclusion cannot be drawn that the peptides do not bind to CD-44 and  $\beta$ 1 considering the fact that osteoprogenitor cells express different integrin receptors at different levels. It would be expected by one skilled in the art that the amount of cell spreading in response to binding to a particular integrin receptor would be proportional to the level of expression of that receptor, if more than one type of receptor is expressed that can bind to the same peptide.

Horton, *Int. J. Biochem. Cell. Biol.* 29(5):721-25 (1997), states that  $\alpha$ v $\beta$ 3 expression has been shown in numerous cell types (see section entitled "Biosynthesis and Tissue Distribution" on page 722). Even though Horton does specifically teach the presence of  $\alpha$ v $\beta$ 3 on stem cells, there are art recognized techniques for determining the integrin expression profile of a cell, and

the integrins expressed by the cells recited in claim 6, such as  $\alpha v \beta 3$  are known in the art. A patent need not teach, and preferably omits, what is well known in the art.

Osseointegration is a complex process and it involves wound healing and osteogenesis. The cells involved in these processes, (such as those listed claim 6) are known in the art, and are defined in the specification (see page 2, line 29 to page 4, line 14, page 15, lines 10-20 and page 29, lines 3-13, ). Because of the ubiquitous expression of integrins on cells, and the fact that the specification clearly demonstrates the ability of the peptides to bind to ***at least one*** integrin a cell surface, claim 6 which recites that the claimed peptides bind to at least one integrin receptor on a cell wherein the cell is selected from the cells listed claim 6, is enabled.

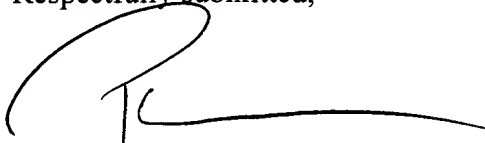
#### (9) SUMMARY AND CONCLUSION

The examiner has provided only speculation and unsupported arguments for why the specification is not enabling. It is well established that a specification is presumed to be enabling. A *prima facie* case of non-enablement can only be made upon a showing of evidence, ***not argument***, of why one skilled in the art would not be able to make and use the claimed subject matter. Even assuming *arguendo* that the examiner has done so, appellant has rebutted this with reference to support not only in the specification but in the literature. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir.1988). It is clear from the direction and guidance given by the specification, the presence of working examples, the state of the prior art and the relative skill of those in the art, that one of ordinary

skill in the art could make the claimed peptides which bind to at least one integrin receptor a cell surface, and use the peptides to increase the cell attachment to a biomaterial and cell spread.

For the foregoing reasons, Appellants submit that claims 1-3, 5 and 6 are enabled.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Patrea L. Pabst', written over a horizontal line.

Patrea L. Pabst  
Reg. No. 31,284

Date: February 24, 2006  
PABST PATENT GROUP LLP  
400 Colony Square, Suite 1200  
1201 Peachtree Street  
Atlanta, Georgia 30361  
(404) 879-2151  
(404) 879-2160 (Facsimile)

### **Appendix: Claims On Appeal**

1. An active osteopontin peptide fragment comprising an amino acid sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein the peptide binds to at least one integrin receptor on a cell surface selected from the group consisting of  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $4 \beta 1$ ,  $2 \beta 1$ , VCAM, ICAM CD44, V3Vx.
2. The peptide fragment of claim 1, wherein the peptide increases cell attachment to a biomaterial and increases cell spread.
3. The peptide fragment of claim 1, wherein the peptide binds to at least one integrin receptor on a cell surface selected from the group consisting of VCAM, ICAM CD44, and V3Vx.
5. The peptide fragment of claim 1 wherein the integrin(s) is selected from the group consisting of  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $4 \beta 1$ , and  $2 \beta 1$ .
6. The peptide fragment of claim 1 wherein the cell is selected from the group consisting of osteoprogenitor cells, tumor cells, macrophages, periosteal cells, endothelial cells, epithelial cells, eosinophils, stem cells, limited potential precursor cells, precursor cells, committed precursor cells, and differentiated cells.

### **Evidence Appendix**

**I. Evidence submitted with Amendment May 11, 2004**

Hu, et al., J. Biol. Chem. 270(44):26232-26238 (1995)

Tuck, et al., J. Cell. Biochem. 78:465-475 (2000)

**II. Evidence submitted with Substitute Appeal Brief December 2, 2004**

Noonan, et al., J. Orthop. Res. 14(4):573-581 (1996) (Abstract)

**III. Evidence submitted with Amendment June 15, 2005**

Webster's Third New International Dictionary definition of "fragment"

Horton, Intl. J. Biochem. 29(5):721-725 (1997)

Blast 2 sequences comparing SEQ ID No: 1 and SEQ ID NO:15

NCBI Conserved Domain Search SEQ ID NO:11, SEQ ID NO:15

## A Biochemical Characterization of the Binding of Osteopontin to Integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$ \*

(Received for publication, May 15, 1995, and in revised form, August 14, 1995)

Dana D. Hu†§, Emme C. K. Lin†¶, Nicholas L. Kovach||, John R. Hoyer\*\*\*, and Jeffrey W. Smith‡

From the ‡Cancer Research Center, Program on Cell Adhesion and the Extracellular Matrix, La Jolla Cancer Research Foundation, La Jolla, California 92037, the ||University of Washington, School of Medicine, Division of Hematology, Seattle, Washington 98195, and the \*\*\*Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

Osteopontin (OPN) is an extracellular matrix protein that binds to integrin  $\alpha_v\beta_3$ . Here we demonstrate that two other integrins,  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , are also receptors for OPN. Human embryonic kidney 293 cells adhere to human recombinant osteopontin (glutathione *S*-transferase-osteopontin; GST-OPN) using integrin  $\alpha_v\beta_1$ . When the 293 cells are transfected with the  $\beta_5$  subunit, they can also adhere to GST-OPN using integrin  $\alpha_v\beta_5$ . Divalent cations regulate the binding of GST-OPN to both  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ .  $Mg^{2+}$  and  $Mn^{2+}$  support the binding of GST-OPN to these integrins but  $Ca^{2+}$  does not. The highest affinity is observed in  $Mn^{2+}$ . In the presence of this ion, the affinity of GST-OPN for  $\alpha_v\beta_1$  is 18 nM and the affinity for  $\alpha_v\beta_5$  is 48 nM. The antibody 8A2, which is an agonist for  $\beta_1$ , promotes the adhesion of 293 cells to GST-OPN even when  $Ca^{2+}$  is present. This observation suggests that cellular events could modulate the affinity of  $\alpha_v\beta_1$  for OPN. Collectively, these findings prove that integrins  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ , and  $\alpha_v\beta_5$  have similar affinity for OPN. Therefore, all three integrins must be considered when evaluating the biological affects of OPN.

Osteopontin (OPN)<sup>1</sup> is a secreted phosphoprotein that was originally isolated from bone (1). OPN is also found in many other fluids and tissues including milk, urine, placenta, kidney, leukocytes, smooth muscle cells, and some tumor cells (for reviews, see Refs. 1 and 2). OPN supports cell adhesion through its Arg-Gly-Asp (RGD) integrin recognition motif. OPN is also rich in aspartic acid residues, and can be heavily glycosylated. The acidic nature of OPN probably accounts for its ability to modulate the growth of calcium crystals in both bone (1, 2) and urine (3).

Integrin  $\alpha_v\beta_3$  is the established receptor for OPN. In bone,  $\alpha_v\beta_3$  is expressed on osteoclasts and it initiates bone resorption by mediating adhesion of the osteoclast to OPN in bone (4–6). It has also been hypothesized that OPN and integrin  $\alpha_v\beta_3$

facilitate vascular remodeling because these two proteins are co-localized in smooth muscle cells following balloon angioplasty (7). Both OPN and integrin  $\alpha_v\beta_3$  are also present in human placenta (8, 9), so their interaction could also be relevant to pregnancy.

Although  $\alpha_v\beta_3$  is clearly a receptor for OPN, many other integrins also bind the RGD motif (10, 11) and no data have excluded other integrins as receptors for OPN. Therefore, we hypothesized that other integrins with the  $\alpha_v$  subunit may also bind OPN. The purpose of this study was to provide a quantitative biochemical analysis of the binding between OPN and integrins  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ . We reason that a measure of these binding affinities will allow a meaningful comparison with the binding affinity of OPN to  $\alpha_v\beta_3$  (12). If more than one integrin does bind OPN with similar affinity, then much information attributing adhesion and signaling events entirely to the interaction between OPN and  $\alpha_v\beta_3$  should be re-evaluated.

### MATERIALS AND METHODS

**Cell Lines**—Human embryonic kidney carcinoma 293 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 10% fetal calf serum (Irvine Scientific), 20 mM Hepes (pH 7.4), 1% glutamine, 1% penicillin, and 1% streptomycin (Sigma). Human integrin subunit  $\beta_5$  was cloned using polymerase chain reaction and subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Kidney 293 cells were transfected at passage 40 with  $\beta_5$ /pcDNA3 or pcDNA3 vector alone using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate transfection reagent (Boehringer Mannheim). Stable transfectants were obtained after selection in 500  $\mu$ g/ml G418 (Sigma) for 2 weeks and maintained thereafter in 250  $\mu$ g/ml G418. Cells expressing high levels of  $\alpha_v\beta_5$  were obtained by sterile FACS with an anti- $\beta_5$  monoclonal antibody (mAb), P3G2.

**Protein Expression and Purification**—In this study a recombinant form of OPN fused the glutathione *S*-transferase (GST-OPN) was used as ligand. We have previously described the characterization of this ligand (12). GST-OPN supports cell adhesion in a manner equivalent to native uropontin, a form of OPN purified from human urine (12). We have also found that both versions of OPN function equally in supporting cell adhesion through integrin  $\alpha_v\beta_5$  and  $\alpha_v\beta_1$  (data not shown). GST-OPN was chosen in the interest of consistency in performing cell binding studies and because of its availability. Integrin  $\alpha_v\beta_5$  was purified from a human placental extract using monoclonal antibody affinity chromatography as described previously (13). The identity and the purity of this protein was assessed by N-terminal amino acid sequencing and by its ability to bind a series of monoclonal antibodies specific for either  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$ .

Vitronectin was purified from human plasma by affinity chromatography on heparin-Sepharose as described (14).

**Antibodies**—The monoclonal antibody 8A2 and its Fab fragment bind to the integrin  $\beta_1$  subunit and stimulate the ligand binding function of integrins containing this subunit. An in-depth characterization of this antibody has been published (15, 16). Monoclonal antibody L230 (anti- $\alpha_v$ ) was purified from cell culture supernatant from hybridoma cells (ATCC, HB8448) by using protein A-Sepharose. The blocking activity of this antibody has been reported previously (17). Monoclonal antibody P4C10 (anti- $\beta_1$ ) was purchased from Life Technologies, Inc. and was used in ascites form, normally at a dilution of 1:500. Anti- $\beta_1$  monoclonal

\* This work was supported in part by National Institutes of Health Grants CA 56483 and AR 42750 (to J. W. S.), DK 10964 (to N. L. K.), and DK 33501 (to J. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported initially by a postdoctoral grant from Monsanto/Searle and then by a postdoctoral fellowship from the California Affiliate of the American Heart Association.

¶ Supported by a postdoctoral grant from the Cancer Research Institute.

|| Established Investigator of the American Heart Association and Genentech.

† The abbreviations used are: OPN, osteopontin; GST-OPN, recombinant osteopontin that is a fusion protein with glutathione *S*-transferase; RGD, Arg-Gly-Asp; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting.

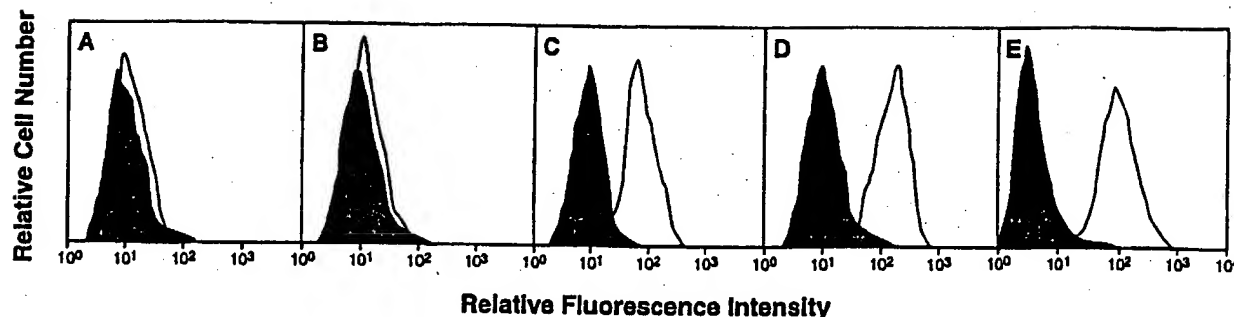


FIG. 1. FACS analysis of integrin expression on kidney 293 cells. A panel of monoclonal antibodies was used to assess integrin expression on wild-type and  $\beta_5$ -transfected human kidney 293 cells. Cells were incubated with mouse IgG or with the noted primary antibodies and then with secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Following extensive washing to remove free antibody the cells were analyzed by flow cytometry. The expression level of each integrin subunit is indicated by the mean fluorescence intensity. The integrin expression profile of wild-type 293 cells was analyzed with mAb LM609 against  $\alpha_v\beta_3$  (A), P3G2 against  $\alpha_v\beta_5$  (B), 14H4 against  $\alpha_v$  (C), and mAb 1977 against  $\beta_1$  (D). Following transfection of these cells with the cDNA for  $\beta_5$  the expression of the  $\alpha_v\beta_5$  heterodimer was detected with mAb P3G2 (E). Cells transfected with the vector pcDNA3 alone exhibited a profile identical to wild-type 293 cells (not shown).

1977 was purchased from Chemicon Int. Inc.. Monoclonal antibody 6B9 (anti- $\alpha_v\beta_3$ ) was produced in this laboratory (18). The polyclonal antibody T545 was raised in this laboratory by immunizing rabbits with highly purified integrin  $\alpha_v\beta_3$ . Prior characterization shows that T545 binds and immunoprecipitates any integrin containing the  $\alpha_v$  subunit (data not shown). Nonspecific mouse IgG was obtained from Calbiochem. Monoclonal antibodies LM609 (anti- $\alpha_v\beta_3$ ) and P3G2 (anti- $\alpha_v\beta_5$ ) were generously provided by Dr. David Cherish (The Scripps Research Institute).

**Synthetic Peptides**—The synthetic peptides with sequence GRGDSP and SPGDRG were purchased from Coast Scientific (La Jolla, CA).

**Fluorescence-activated Cell Sorting (FACS)**—FACS analysis was performed using standard protocols. Briefly, cells were harvested in phosphate-buffered saline/EDTA, washed once with Dulbecco's modified Eagle's medium, and resuspended in the same media. Cells were incubated with primary antibody for 30 min on ice and then washed twice. Cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Caltag) for 30 min on ice. Cells were washed twice with media and resuspended in phosphate-buffered saline for FACS analysis. FACS analysis was performed on a Becton Dickinson FACSort.

**Cell Adhesion Assays**—Cell adhesion was measured as described previously (19). GST-OPN or vitronectin were coated onto 96-well microtiter plates (Titertek) and incubated overnight at 4 °C. Our measurements using  $^{125}$ I-GST-OPN as a tracer indicate that 13–19% of the GST-OPN actually binds the plate when the coating concentration is between 1 and 100 nM. Thus, the amount of ligand available for cell adhesion is considerably less than the coating concentration. There was little variability in coating efficiency so comparisons of cell adhesion as a function of coating concentration are valid. Following exposure to GST-OPN, the plates were then blocked by 30 mg/ml bovine serum albumin in TBS (pH 7.4) for 1 h at 37 °C. Cells were harvested from tissue culture flasks with phosphate-buffered saline/EDTA, washed, and resuspended in adhesion buffer containing 1 × Hanks' balanced salt solution lacking divalent cations, 50 mM Hepes (pH 7.4), 1 mg/ml bovine serum albumin and 0.5 mM  $Mn^{2+}$ , 2 mM  $Ca^{2+}$ , or 2 mM  $Mg^{2+}$ . In most experiments 100  $\mu$ l of cells ( $1.5 \times 10^6$  cells/ml) were added to each well. Where required, appropriate concentration of agonists (e.g. activating mAb 8A2, typically at 1  $\mu$ g/ml) or antagonists (e.g. EDTA at 20 mM or blocking antibodies, 1:500 for ascites and 5–20  $\mu$ g/ml for purified mAbs) were mixed with the cells before they were added to the wells. Various batches of control ascites gave no inhibition at a 1:500 dilution. After a 45-min incubation at 37 °C, the non-adherent cells were washed off with TBS by gentle aspiration. Adherent cells were detected by a colorimetric assay measuring endogenous cellular lysosomal acid phosphatase activity with a chromophore that absorbs at 405 nm (20). A standard curve with cells in suspension showed that absorbance values were directly proportional to cell number. All experiments were performed at least three times yielding identical results.

**Radioligand Binding Measurements**—To assess the affinity of GST-OPN for integrins on the 293 cells, binding isotherms of the interaction between  $^{125}$ I-labeled GST-OPN and 293 cells were generated. GST-OPN was radiolabeled with  $Na^{125}I$  using IODO-GEN (Pierce Chemical Co.). The specific activity was between 2 and  $7 \times 10^{-4}$  cpm/ng of protein. For binding assays, cells were harvested and resuspended in adhesion buffer containing 0.5 mM  $Mn^{2+}$ , which had been found to promote

maximal cell adhesion to GST-OPN. A concentration range of  $^{125}$ I-GST-OPN was added to the 293 cells or the  $\beta_5$ -transfected 293 cells ( $1 \times 10^6$  cells/ml) in suspension and the mixture was then incubated for 70 min at 14 °C. At the end of the incubation period, quadruplicate samples of cells (90  $\mu$ l) were carefully layered onto 20% sucrose cushions (280  $\mu$ l) in microfuge tubes (West Coast Scientific Inc., Hayward, CA). The tubes were centrifuged for 3 min at 14,000 rpm and the cell pellet in the tip of the tube was amputated and counted in a  $\gamma$ -counter. Nonspecific binding was measured in the presence of 20 mM EDTA and was subtracted from total binding to yield specific binding. All measurements were repeated at least three times yielding identical results.

Bound protein was calculated from the specific activity of the labeled ligand and the results are presented as molecules bound per cell,  $[GST-OPN]_{bound}$ . Scatchard plots were derived by plotting  $\gamma/[GST-OPN]_{free}$  against  $\gamma$ , where  $\gamma$  represents  $[GST-OPN]_{bound}$ /total number of cells. The binding affinity ( $K_d$ ) of cell surface integrin for GST-OPN is derived from the slope of this plot. In cases where blocking antibodies were present, preincubation with the antibodies at 14 °C for 15 min was carried out prior to adding  $^{125}$ I-GST-OPN. In cases where binding was stimulated with 8A2, the antibody was added simultaneously with the labeled ligand.

The ability of purified integrin  $\alpha_v\beta_5$  to bind GST-OPN was also measured using a solid phase binding assay previously described (19). Purified  $\alpha_v\beta_5$  was immobilized on 96-well Titertek microtiter plates at a coating concentration of 50 ng/well. After incubation overnight at 4 °C, nonspecific protein binding sites on the plate were blocked with 30 mg/ml bovine serum albumin and 1 mM of the desired divalent cation(s) in TBS (pH 7.4). Radiolabeled GST-OPN in either 2 mM  $Ca^{2+}$  or 0.2 mM  $Mn^{2+}$  was then added to the plate. In control wells, nonspecific binding was measured in the presence of a competing RGD peptide. Nonspecific binding was subtracted from the total binding to yield specific binding. Each data point is a result of the average of triplicate wells.

## RESULTS

**Generating Cell Lines to Study the Binding between OPN and  $\alpha_v$ -Integrins**—To study the binding of  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  to OPN we chose the kidney 293 cells because they lack the  $\alpha_v\beta_3$  integrin. These cells do express endogenous  $\alpha_v\beta_1$  (21). Thus, the wild-type 293 cells serve as a model for measuring OPN binding to  $\alpha_v\beta_1$ . To generate a cell line with which we could measure the interaction of  $\alpha_v\beta_5$  with OPN, the 293 cells were transfected with the cDNA for  $\beta_5$ . The integrin profile of the wild-type and  $\beta_5$ -transfected 293 cells was compared by flow cytometry (Fig. 1). These studies confirm that the wild-type 293 cells fail to express  $\alpha_v\beta_3$  (panel A) or  $\alpha_v\beta_5$  (panel B). The cells express both the  $\alpha_v$  and  $\beta_1$  subunits (panels C and D). Our immunoprecipitations are consistent with prior studies (21, 22) which indicate that  $\alpha_v\beta_1$  is the predominant  $\beta_1$  containing integrin on these cells (data not shown). Following transfection with the  $\beta_5$  cDNA the 293 cells also express the  $\alpha_v\beta_5$  heterodimer on the cell surface (panel E). The cells transfected with the cDNA for  $\beta_5$  display a 10-fold greater binding of anti- $\beta_5$  antibody than the vector transfected or wild-type 293 cells. The expression of



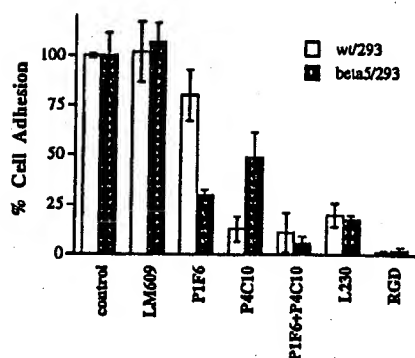


FIG. 2. Wild-type 293 cells and  $\beta_5$ -transfected 293 cells adhere to OPN. The adhesion of wild-type (open bars) and  $\beta_5$ -transfected (dark bars) 293 cells to GST-OPN was challenged with a series of blocking monoclonal antibodies. Cell adhesion to GST-OPN was performed in the presence of LM609 (anti- $\alpha_v\beta_3$ ), P1F6 (anti- $\alpha_v\beta_5$ ), P4C10 (anti- $\beta_1$ ), the mixture of P1F6 and P4C10, L230 (anti- $\alpha_v$ ), and RGD peptide as a control inhibitor. The results are expressed as a percentage of control adhesion in the presence of mouse IgG (control). The data are the mean of triplicate wells. Error bars denote the standard deviation. This experiment was performed four times yielding identical results.

$\alpha_v\beta_5$  on 293 cells was also confirmed by immunoprecipitation and Western blotting using antibodies specific for  $\alpha_v\beta_5$  (data not shown).

**Integrins  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  are Receptors for OPN**—To determine whether  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  could mediate cell adhesion to GST-OPN, the wild-type and  $\beta_5$ -transfected 293 cells were allowed to adhere to immobilized GST-OPN. Both cell lines adhere to GST-OPN (Fig. 2). The adhesion was blocked by RGD peptide and by P4C10, an antibody against the  $\beta_1$  subunit. The adhesion of these cells was also inhibited by L230, an antibody that blocks function of  $\alpha_v$ . The antibody against  $\alpha_v\beta_3$ , LM609, had no effect. Based on these data, and immunoprecipitation experiments showing that the majority of  $\beta_1$  in these cells is complexed with  $\alpha_v$  (data not shown), we conclude  $\alpha_v\beta_1$  is a receptor for OPN.

The adhesion of  $\beta_5$ -transfected 293 cells was also blocked by the antibody against the  $\alpha_v$  subunit (L230). Approximately 70% of the adhesion of the  $\beta_5$ -transfected cells could be blocked by P1F6, an antibody that interferes with ligand binding to  $\alpha_v\beta_5$ . The remainder of the adhesion (30%) could be blocked by antibody against the  $\beta_1$  subunit, indicating that the endogenous  $\alpha_v\beta_1$  contributes to the adhesion of these cells to OPN. These experiments show that  $\alpha_v\beta_5$  can also mediate cell adhesion to OPN.

**The Cation Dependence of Adhesion to OPN Is Distinct from the Cation Dependence for Vitronectin**— $\text{Ca}^{2+}$  does not support the binding of OPN to integrin  $\alpha_v\beta_3$  (12). To determine if  $\text{Ca}^{2+}$  is similarly ineffective in supporting GST-OPN binding to  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , we tested the ability of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$  to support the adhesion of wild-type and  $\beta_5$ -transfected 293 cells to GST-OPN (Fig. 3, panels A and B). For comparison, the ability of each ion to support the adhesion of each cell line to vitronectin is also shown (panels C and D). In this study, the amount of coated protein was varied across a concentration range. Each ion was used at a concentration found to support maximal adhesion (not shown).  $\text{Ca}^{2+}$  did not support adhesion of either cell line to GST-OPN. However,  $\text{Ca}^{2+}$  did enable maximal cell adhesion to vitronectin.  $\text{Mn}^{2+}$  was most effective in supporting the adhesion of  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  expressing cells to GST-OPN.  $\text{Mg}^{2+}$ , which is likely to be the physiologically relevant ion, also supported adhesion. Despite slight differences in the rank order potency of divalent ions in supporting adhesion to vitronectin, all three ions did support maximal adhesion to this protein. Physiologic levels of  $\text{Ca}^{2+}$  supported adhesion to

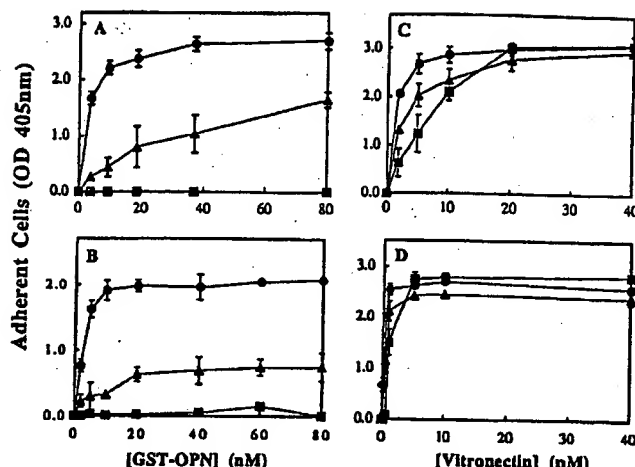


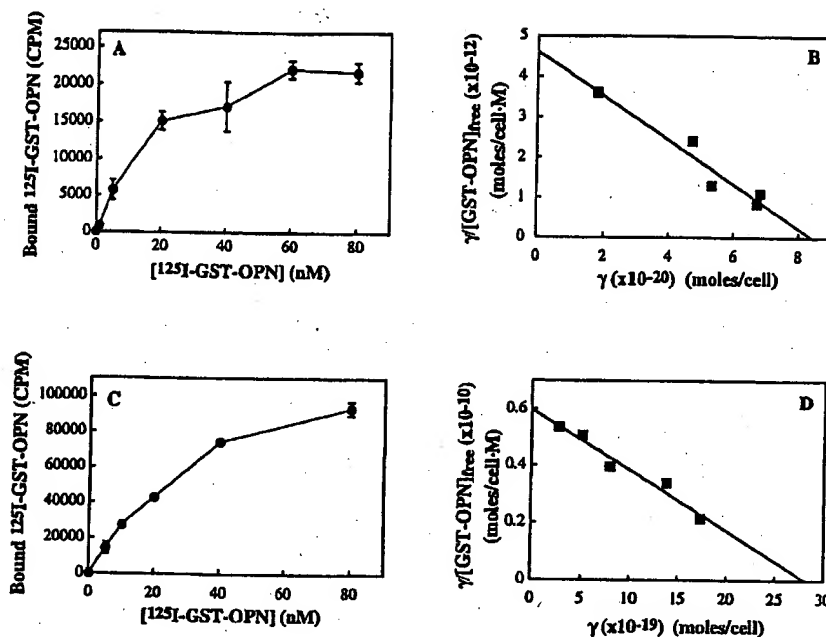
FIG. 3. A comparison of the effects of divalent ions on cell adhesion to osteopontin and vitronectin. The adhesion of kidney 293 cells expressing either integrin  $\alpha_v\beta_1$  (panels A and C) or integrin  $\alpha_v\beta_5$  (panels B and D) to either GST-OPN (panels A and B) or vitronectin (panels C and D) was tested in buffer containing  $\text{Ca}^{2+}$  (■),  $\text{Mg}^{2+}$  (□), or  $\text{Mn}^{2+}$  (●). The adhesion of the  $\beta_5$ -transfected cells was measured in the presence of antibody P4C10 to eliminate any contribution of endogenous  $\alpha_v\beta_1$  to cell adhesion. Adhesion assays were conducted as described under "Experimental Procedures." Each data point is the average of quadruplicate measurements. This experiment was performed four times yielding identical results. Additionally, in separate experiments, identical results were obtained when uropontin was used as immobilized ligand.

vitronectin but not to GST-OPN. We conclude that there is a fundamental difference in the cation requirement of integrin binding to OPN as opposed to vitronectin.

**Measuring the Affinity of GST-OPN for  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$** —The OPN receptor that has received the most attention is integrin  $\alpha_v\beta_3$ . We recently measured the affinity between GST-OPN and purified integrin  $\alpha_v\beta_3$  and found the apparent  $K_d$  to be between 5 and 30 nM (12). Recent binding studies between GST-OPN and  $\alpha_v\beta_3$  on the surface of M21 melanoma cells has yielded a similar affinity (data not shown). To gauge the significance of the binding of OPN to  $\alpha_v\beta_1$  or  $\alpha_v\beta_5$ , it is important to compare the binding affinities between OPN and each of these integrins. To measure the affinity of OPN for  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , we performed binding assays with soluble  $^{125}\text{I}$ -GST-OPN. These binding studies were performed by harvesting the wild-type 293 cells or the  $\beta_5$ -transfected 293 cells from tissue culture flasks and placing the cells in suspension. Binding studies were done in  $\text{Mn}^{2+}$  to obtain the highest affinity between GST-OPN and the two integrins. In the case of the  $\beta_5$ -transfected cells, we found that a small component (typically 10–20% of total binding) of GST-OPN binding was mediated through endogenous  $\alpha_v\beta_1$ . To eliminate this component from the analysis, the binding studies with the  $\beta_5$ -transfected cells were performed in the presence of a saturating level of a function blocking antibody against the  $\beta_1$  subunit. Initial control binding studies showed that the specific binding of  $^{125}\text{I}$ -GST-OPN to both wild-type and  $\beta_5$ -transfected 293 was inhibited completely by an RGD peptide and by blocking antibody against the  $\alpha_v$  subunit (data not shown). To measure the relative affinity of GST-OPN for integrin  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , binding isotherms were generated across a concentration range of  $^{125}\text{I}$ -GST-OPN (Fig. 4). Scatchard analysis of the binding isotherms revealed that OPN has an affinity of 18 nM for  $\alpha_v\beta_1$  (Fig. 4B) and 48 nM for  $\alpha_v\beta_5$  (Fig. 4D). These affinity constants are similar to the apparent  $K_d$  (5–30 nM) we measured between GST-OPN and purified  $\alpha_v\beta_3$  (12). Consequently, the binding affinity between GST-OPN and all three  $\alpha_v$ -integrins is similar.

**Binding of GST-OPN to Purified Integrin  $\alpha_v\beta_5$** —Integrin

FIG. 4. A measurement of the binding affinity between GST-OPN and  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ . Isotherms of  $^{125}\text{I}$ -GST-OPN binding to wild-type 293 cells (A) and  $\beta_5$ -transfected 293 cells (C) maintained in suspension were generated. Cells were harvested from tissue culture flasks as usual and were resuspended in adhesion buffer containing 0.5 mM  $\text{Mn}^{2+}$ .  $\text{Mn}^{2+}$  was chosen to measure the highest affinity between GST-OPN and the two integrins.  $^{125}\text{I}$ -GST-OPN of increasing concentration was added to the cells and the mixture was allowed to incubate with rocking for 70 min at 14 °C. Bound ligand was separated from free ligand by centrifugation through sucrose cushions (see "Experimental Procedures"). Each point is the average of triplicate data points and each isotherm is representative of at least three repetitions. The error bars show the standard deviation. To derive the affinity of the interaction between GST-OPN and integrin  $\alpha_v\beta_1$  or integrin  $\alpha_v\beta_5$  the data shown in panels A and C were replotted according to the method of Scatchard (53). This derivation yields Scatchard plots for GST-OPN binding to  $\alpha_v\beta_1$  (B) and  $\alpha_v\beta_5$  (D). The  $R^2$  values for these lines are 0.87 and 0.90, respectively.



$\alpha_v\beta_5$  is abundant enough in placenta to purify  $\alpha_v\beta_5$  for direct binding studies (13). We measured the binding of  $^{125}\text{I}$ -GST-OPN to purified  $\alpha_v\beta_5$  using the same format that was previously used for  $\alpha_v\beta_3$  (12). As shown in Fig. 5A,  $\text{Mn}^{2+}$  is more effective than  $\text{Ca}^{2+}$  in promoting the binding of OPN to  $\alpha_v\beta_5$ . Although this assay format does not allow an exact derivation of  $K_d$  because the binding of ligand to integrin is irreversible in this assay format (11), we can assign an apparent  $K_d$  and compare this value to that obtained for  $\alpha_v\beta_3$ . In  $\text{Mn}^{2+}$ , the apparent  $K_d$  of GST-OPN for  $\alpha_v\beta_5$  is 20 nM, which is comparable to the value of 5–30 nM for  $\alpha_v\beta_3$  (12). Thus, the two purified integrins bind GST-OPN with nearly equal affinity. The purified  $\alpha_v\beta_5$  is obtained from a placental lysate by first depleting the lysate of  $\alpha_v\beta_3$  by affinity chromatography. Therefore, we performed an enzyme-linked immunosorbent assay on the purified  $\alpha_v\beta_5$  to make sure that it contained no contaminating  $\alpha_v\beta_3$ . This enzyme-linked immunosorbent assay was done with mAb 6B9 which is specific for  $\alpha_v\beta_5$  (18) and mAb LM609 which binds only to  $\alpha_v\beta_3$ . As shown in Fig. 5B, the purified  $\alpha_v\beta_5$  contains no detectable  $\alpha_v\beta_3$ , proving that OPN binds to purified  $\alpha_v\beta_5$ .

#### Adhesion to OPN through Integrin $\alpha_v\beta_1$ Can Be Stimulated by Activation of the $\beta_1$ Subunit with Monoclonal Antibody 8A2

It has been reported that many integrins can exist in multiple affinity states (16, 24–30). These observations indicate that there may be cellular pathways that control the affinity of an integrin for its ligand. Because our data shows that  $\text{Ca}^{2+}$  does not support adhesion to OPN, we wondered if other stimuli could override this phenomena. Since the physiologic stimuli that regulate integrin affinity have not been completely discerned, we made use of the monoclonal antibody 8A2. This antibody is a known agonist for  $\beta_1$  integrins (15, 16) and it has been suggested that 8A2 mimics the physiologic activation of these integrins. We tested the ability of 8A2 to stimulate the adhesion of 293 cells to OPN. These studies were performed in buffer containing  $\text{Ca}^{2+}$ . As shown in Fig. 6A, 293 cells adhered to GST-OPN in the presence of mAb 8A2 in buffer containing  $\text{Ca}^{2+}$ . No adhesion to GST-OPN was observed in the presence of normal mouse IgG in the same buffer. To determine whether this stimulation was saturable and corresponded with the binding of 8A2 to  $\alpha_v\beta_1$ , the number of cell surface binding

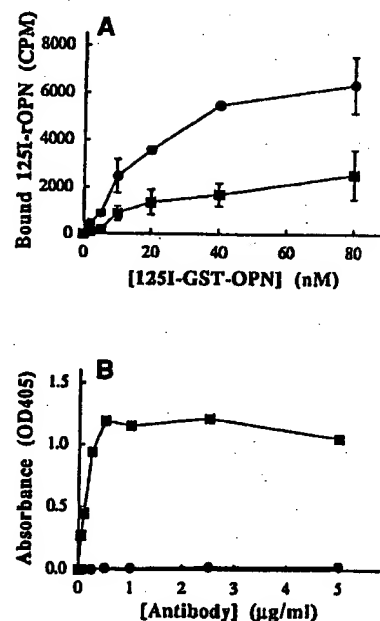


FIG. 5. Integrin  $\alpha_v\beta_5$  is also a receptor of osteopontin. A, the binding of GST-OPN to integrin  $\alpha_v\beta_5$  was also determined by a solid phase binding assay. This study was done in buffer containing  $\text{Mn}^{2+}$  (0.2 mM, ●) or  $\text{Ca}^{2+}$  (2 mM, ■) as divalent cation. The binding assay was performed as described previously (19). The data are the average of triplicate points in which the error was less than 12% of the total binding. Nonspecific binding was less than 8% of the total binding as determined by incubation with competing RGD peptide. Nonspecific binding is subtracted from the total binding. B, to ensure that no contaminating  $\alpha_v\beta_3$  was present in the  $\alpha_v\beta_5$  preparation, an enzyme-linked immunosorbent assay was performed. The monoclonal antibody 6B9 (■) (18) was used as a probe of integrin  $\alpha_v\beta_5$  and antibody LM609 (●) was used to detect integrin  $\alpha_v\beta_3$ .

sites for the antibody was measured. As shown in Fig. 6B the binding of  $^{125}\text{I}$ -mAb 8A2 to 293 cells in suspension approaches saturation between 0.5 and 1  $\mu\text{g/ml}$  of antibody. This concentration corresponds closely with the amount of the antibody that maximally stimulates adhesion to OPN (Fig. 6A). From the Scatchard plot shown in Fig. 6C, the  $K_d$  of mAb 8A2 for  $\alpha_v\beta_1$

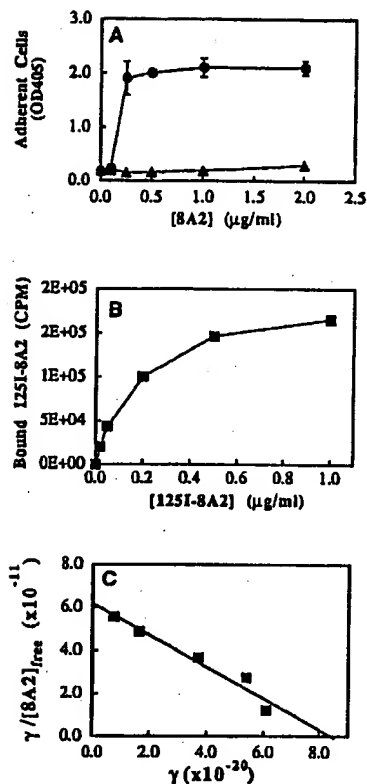


FIG. 6. Antibody 8A2 stimulates 293 cell adhesion to OPN in  $Ca^{2+}$ . A, the adhesion of wild-type 293 cells to GST-OPN was measured in the presence of a range of mAb 8A2 (●) or normal mouse IgG (○). Cells were resuspended in adhesion buffer containing 2 mM  $Ca^{2+}$ . The cells (100  $\mu$ l at  $1.5 \times 10^6$  cells/ml) were allowed to adhere to GST-OPN at a coating concentration of 10 nM. The data are the mean of triplicate wells. Error bars denote the standard deviation. This experiment was performed three times yielding identical results. B, the affinity and number of binding sites on 293 cells for mAb 8A2 was measured by generating a binding isotherm with radiolabeled 8A2. Nonspecific binding was determined by competition with an excess of unlabeled 8A2 and was typically less than 10% of total binding. The specifically bound counts are shown. C, these data were transformed into a Scatchard plot (53) to quantify the binding affinity and the number of binding sites.

on 293 cells is 1.4 nM and the number of cell surface binding sites is 51,000. This value matches exactly the number of  $\alpha_v\beta_1$  molecules on the cell surface as measured by binding of <sup>125</sup>I-GST-OPN (Fig. 4, A and B).

We also examined the ability of mAb 8A2 to stimulate cell adhesion across the range of coated GST-OPN (Fig. 7A). In the presence of mAb 8A2, the coating concentration of GST-OPN that support half-maximal cell adhesion is similar to that obtained in  $Mn^{2+}$  (Fig. 3A), indicating that both 8A2 and  $Mn^{2+}$  induce the high affinity state of  $\alpha_v\beta_1$ . To verify that mAb 8A2 stimulates adhesion to OPN by enhancing the affinity state of  $\alpha_v\beta_1$ , adhesion assays were done in the presence of mAb 8A2 and a series of antagonists, including RGD peptide, antibody P4C10 (anti- $\beta_1$ ), and mAb L230 (anti- $\alpha_v$ ). The adhesion to GST-OPN that is induced by mAb 8A2 can be blocked by each of the above inhibitors (Fig. 7B). Neither random peptide nor mouse IgG affected cell adhesion. Several other control experiments were also performed. These studies showed that the Fab fragment of mAb 8A2 was as effective as the whole antibody and that mAb 8A2 did not induce the expression of more  $\alpha_v\beta_1$  on the cell surface.

#### DISCUSSION

Many interactions between cells and the extracellular matrix depend on cellular recognition of the RGD motif within

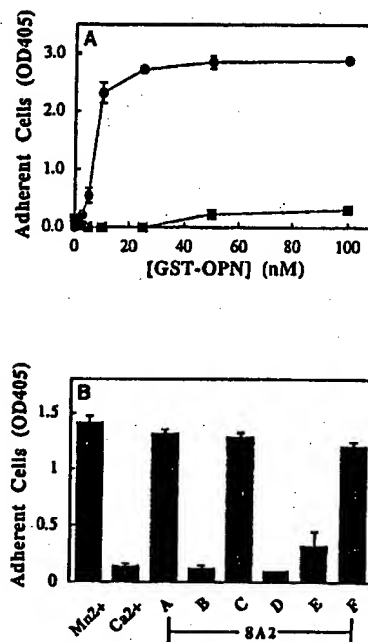


FIG. 7. mAb 8A2 stimulates adhesion to GST-OPN through integrin  $\alpha_v\beta_1$ . A, the adhesion of wild-type 293 cells to GST-OPN was measured in the presence of a range of coated GST-OPN in the presence of 1  $\mu$ g/ml of either 8A2 (●) or normal mouse IgG (○). Cells (100  $\mu$ l at  $1.5 \times 10^6$  cells/ml) were resuspended in adhesion buffer containing 2 mM  $Ca^{2+}$  and were allowed to adhere to a range of GST-OPN for 45 min at 37 °C. The data are the mean of triplicate wells. Error bars denote the standard deviation. B, to confirm that integrin  $\alpha_v\beta_1$  is mediating 8A2-stimulated adhesion to GST-OPN in  $Ca^{2+}$ , the adhesion was challenged by synthetic peptides and monoclonal antibodies. These are: mAb 8A2 only (A), 100  $\mu$ M GRGDSP (B), 100  $\mu$ M SPDGRG (C), 1:500 dilution of anti- $\beta_1$  ascites P4C10 (D), 20  $\mu$ g/ml of anti- $\alpha_v$  mAb L230 (E), and 20  $\mu$ g/ml nonspecific mouse IgG (F).

adhesive proteins. Small peptides with the RGD sequence will bind to several integrin adhesion receptors, but larger adhesive proteins display considerable integrin binding specificity. Therefore, an important issue with every RGD-containing adhesive protein is to identify its receptor(s). OPN, for instance, binds to integrin  $\alpha_v\beta_3$ , but not to the platelet integrin  $\alpha_{IIb}\beta_3$  (12). However, it is now apparent that several integrins have ligand binding properties similar to  $\alpha_v\beta_3$ , these are the four other integrins containing the  $\alpha_v$  subunit,  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$ ,  $\alpha_v\beta_6$ , and  $\alpha_v\beta_8$  (22). Like  $\alpha_v\beta_3$ , two of these integrins,  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , bind to vitronectin. This functional similarity lead us to suspect that both of these integrins may also bind OPN. Since both  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  have been identified in tissues, like bone and the vasculature where OPN is involved in tissue remodeling (1, 2, 31), there is the potential for a physiologically relevant interaction between these integrins and OPN.

Ideally experiments designed to characterize the interactions between integrins and their ligands would provide a quantitative measure of these interactions so that a hierarchy of binding affinities is available. Here, the affinity between OPN and integrin  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  was determined by measuring the binding of <sup>125</sup>I-GST-OPN to these integrins present on the surface of kidney 293 cells. Scatchard analysis shows that in the highest affinity state, the  $K_d$  of GST-OPN is 18 nM for  $\alpha_v\beta_1$  and 48 nM for  $\alpha_v\beta_5$ . We also measured the apparent affinity between GST-OPN and purified integrin  $\alpha_v\beta_5$ . It was impossible to determine a  $K_d$  using Scatchard analysis because GST-OPN binding to  $\alpha_v\beta_5$  immobilized in microtiter wells was non-dissociable. This non-dissociable binding has been observed previously with integrin  $\alpha_v\beta_3$  and its potential physiologic significance has been discussed (23). Despite this binding anom-

ally, the apparent  $K_d$  (20 nM) between GST-OPN and purified integrin  $\alpha_v\beta_5$  is comparable to the affinity between GST-OPN and purified  $\alpha_v\beta_3$  measured in the same assay under the same conditions (12). In addition, several cell adhesion experiments showed that the coating concentration of GST-OPN necessary for half-maximal cell adhesion through  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$  (Fig. 3, A and B), and  $\alpha_v\beta_3$  (12) was identical. Collectively, our data suggest there is no substantial preference in the binding of OPN to any of these  $\alpha_v$ -integrins. It is important to reiterate that OPN does not bind to all integrins. We recently measured the binding of OPN to the platelet integrin  $\alpha_{IIb}\beta_3$  and showed that these two proteins do not interact (12).

The binding of OPN to its different  $\alpha_v$ -integrin receptors is also similar with respect to divalent ion requirement. We previously found that both  $Mg^{2+}$  and  $Mn^{2+}$  support OPN binding to integrin  $\alpha_v\beta_3$ , but that  $Ca^{2+}$  suppresses this interaction (12). Here, we show that  $Ca^{2+}$  also fails to support the binding of OPN to integrins  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ . This observation is important because it illustrates a key difference between the binding of OPN and vitronectin to  $\alpha_v$ -integrins. Although small differences exist in the rank-order potency of divalent ions in supporting adhesion to vitronectin, physiologic levels of  $Ca^{2+}$  supported maximal cell adhesion to this protein through  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ . This is in contrast to the adhesion to OPN which is not supported at any level by  $Ca^{2+}$ . In this regard it is worth noting an important biochemical distinction between vitronectin and OPN. The vitronectin used in these studies is a multimer, often containing between 12 and 15 vitronectin moieties per multimer (32, 33). There is substantial evidence that the multimeric vitronectin is also present in extracellular matrices *in vivo* (32–34). In contrast, the OPN used in these studies was proven to be monomeric by mass spectral analysis (12) and gel filtration chromatography (data not shown). The soluble OPN found in body fluids is also assumed to be a monomer. Consequently, it is possible that multimeric vitronectin engages several integrins simultaneously, thereby overriding an otherwise lower affinity between vitronectin and  $\alpha_v$ -integrins in calcium ion.

While  $Ca^{2+}$  does not support OPN binding to integrins  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ ,  $Mn^{2+}$  is able to enhance the binding. This result is not unexpected because  $Mn^{2+}$  is known to activate ligand binding functions of many integrins (22, 35–38). The physiologic activation of integrins can also be mimicked by monoclonal antibodies (16, 39–41). For example, several studies have demonstrated that integrins can be subject to physiologic activation. The best example is the platelet fibrinogen receptor integrin  $\alpha_{IIb}\beta_3$ , which exists in a dormant state on resting platelets. This integrin responds to platelet activation by increasing its affinity for soluble fibrinogen (42). This increased binding affinity enables platelet aggregation at the site of a wound. Our data indicate that the binding of GST-OPN to integrin  $\alpha_v\beta_1$  can be enhanced by both  $Mn^{2+}$  and the mAb 8A2, which is known to be an agonist of other  $\beta_1$ -integrins. Although several other integrins are known to have agonists other than divalent ions (16), to our knowledge, this is the first demonstration that the affinity of an  $\alpha_v$ -integrin can be modulated by an agonist besides  $Mn^{2+}$ . By analogy with other integrins that are similarly stimulated, it is possible that this artificial stimulus indicates the potential for enhancing the affinity state of the integrin by physiologic means. It is important to emphasize that even when  $Ca^{2+}$  is present, the mAb 8A2 was able to enhance cell adhesion to OPN to maximal levels. Thus, the suppressive effects of  $Ca^{2+}$  can be overridden by other stimuli. In future studies, it will be important to determine if  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  can be similarly stimulated to bind OPN when  $Ca^{2+}$  is present and to determine if there are cellular signals that can promote adhesion to OPN in  $Ca^{2+}$ .

The binding of OPN to  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  may be important to bone homeostasis. OPN is thought to be one of the most important matrix proteins for osteoclast adhesion (2, 4). In addition, soluble OPN stimulates intracellular signaling in osteoclasts, including  $Ca^{2+}$  fluxes and the phosphorylation of intracellular proteins (43). It has been reported that integrin  $\alpha_v\beta_1$  is present on human osteoclasts (44–47) and that integrin  $\alpha_v\beta_5$  is present on chicken osteoclast precursors (48, 49). Therefore both of these integrins are positioned to mediate interactions between OPN and cells in bone. Our finding that integrins  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  have high affinity for OPN indicates that interactions between OPN and these receptors may play an essential role in bone remodeling. Blocking the activity of  $\alpha_v\beta_3$  with antibodies inhibits bone resorption, but no analogous study has been done with antagonists of other  $\alpha_v$ -integrins. Our data suggest that similar experiments should be done with antagonists of  $\beta_1$  and  $\beta_5$ .

Recent study also indicates that OPN is involved in vascular injury and repair (6, 31). One of the initial responses to vascular injury is the formation of a neointima which precedes the formation of atherosclerotic lesions (50). Giachelli *et al.* (51) recently showed that OPN expression is increased substantially in both rat and human smooth muscle cells surrounding a vessel that has been exposed to a catheter-induced injury. Because of the temporal regulation of OPN synthesis following this insult, the hypothesis was put forth that the OPN expressed by smooth muscle cells may be an important modulator of cell migration and proliferation associated with neointima formation (7, 52). The same group showed that, integrin  $\alpha_v\beta_3$  mediates only a portion of smooth muscle cell or to OPN; a major component of this adhesion was not blocked by antagonists specific for  $\alpha_v\beta_3$  (7). The data presented in this report indicate that integrins  $\alpha_v\beta_5$  and  $\alpha_v\beta_1$  should be considered as candidate OPN receptors involved in guiding vascular repair.

The kinetic data in this report provide information essential to an understanding of the biology of OPN. Many adhesive and signaling events are tied to cellular exposure to OPN. In large part, it had been assumed that these events are mediated by integrin  $\alpha_v\beta_3$  because it was the only known OPN receptor. In conjunction with our prior study (12), the data in this report show that  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$ , and  $\alpha_v\beta_5$  have similar affinities for OPN and that the ion regulation of OPN binding to each integrin is nearly identical. Therefore, along with  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  must now be considered receptors for OPN.

## REFERENCES

- Butler, W. T. (1989) *Connect. Tissue Res.* 23, 123–136
- Denhardt, D. T., and Guo, X. (1993) *FASEB J.* 7, 1475–1483
- Shiraga, H., Min, W., VanDusen, W. J., Claymen, M. D., Miner, D., Terrell, C. H., Sherbotle, J. R., Foreman, J. W., Przysiecki, C., Neilson, E. G., and Hoyer, J. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 426–430
- Miyachi, A., Alvarez, J., Greenfield, E. M., Teti, A., Grano, M., Colucci, S., Zamboni-Zallone, A., Ross, F. P., Teitelbaum, S. L., Cheresch, D., and Hruska, K. A. (1991) *J. Biol. Chem.* 266, 20369–20374
- Horton, M. A., and Davies, J. (1989) *J. Bone Miner. Res.* 4, 803–808
- Ross, F. P., Chappel, J., Alvarez, J. I., Sander, D., Butler, W. T., Farach-Carson, M. C., Mintz, K. A., Robey, P. G., Teitelbaum, S. L., and Cheresch, D. A. (1993) *J. Biol. Chem.* 268, 9901–9907
- Lilaw, L., Almeida, M., Hart, C. E., Schwartz, S. M., and Giachelli, C. M. (1994) *Circ. Res.* 74, 214–224
- Young, M. F., Kerr, J. M., Termine, J. D., Wewer, U. M., Wang, M. G., McBride, O. W., and Fisher, L. W. (1990) *Genomics* 7, 491–502
- Smith, J. W., and Cheresch, D. A. (1988) *J. Biol. Chem.* 263, 18726–18731
- Albelda, S. M., and Buck, C. A. (1990) *FASEB J.* 4, 2868–2880
- Hynes, R. O. (1992) *Cell* 69, 11–25
- Hu, D. D., Hoyer, J. R., and Smith, J. W. (1995) *J. Biol. Chem.* 270, 9917–9925
- Smith, J. W., Vestal, D. J., Irwin, S. V., Burke, T. A., and Cheresch, D. A. (1990) *J. Biol. Chem.* 265, 11008–11013
- Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988) *Cell Struct. Funct.* 13, 281–292
- Wayner, E. A., and Kovach, N. L. (1992) *J. Cell Biol.* 116, 489–497
- Kovach, N. L., Carlos, T. M., Yee, E., and Harlan, J. M. (1992) *J. Cell Biol.* 116, 499–509
- Weinacker, A., Chen, A., Agrez, M., Cone, R. I., Nishimura, S., Wayner, E., Pytela, R., and Sheppard, D. (1994) *J. Biol. Chem.* 269, 6940–6948
- Stulver, I. S., and Smith, J. W. (1995) *Hybridoma*, in press
- Smith, J. W., Plotrowicz, R. S., and Mathis, D. M. (1994) *J. Biol. Chem.* 269,

- 960-967
20. Pratner, C. A., Plotkin, J., Jaye, D., and Frazier, W. A. (1991) *J. Cell Biol.* 112, 1031-1040
  21. Bodary, S. C., and McLean, J. W. (1990) *J. Biol. Chem.* 265, 5938-5941
  22. Busk, M., Pytela, R., and Sheppard, D. (1992) *J. Biol. Chem.* 267, 5790-5796
  23. Orlando, R. A., and Cheresch, D. A. (1991) *J. Biol. Chem.* 266, 19543-19550
  24. Collier, B. S. (1985) *J. Clin. Invest.* 76, 101
  25. Shimizu, S., van Seventer, G. A., Morgan, K. J., and Shaw, S. (1990) *Nature* 345, 250-253
  26. Wilkins, J. A., Stupack, D., Stewart, S., and Calcia, S. (1991) *Eur. J. Immunol.* 21, 517-522
  27. Saudek, V., Atkinson, R. A., and Pelton, J. T. (1991) *Biochemistry* 30, 7369-7372
  28. Masumoto, A., and Hemler, M. E. (1993) *J. Biol. Chem.* 268, 228-234
  29. Phillips, D. R., Charo, I. F., and Scarborough, R. M. (1991) *Cell* 65, 359-362
  30. Frelinger, A. L., III, Du, X., Plow, E. F., and Ginsberg, M. H. (1991) *J. Biol. Chem.* 266, 17106-17111
  31. Giachelli, C., Bae, N., Lombardi, D., Majesky, M., and Schwartz, S. (1991) *Biochem. Biophys. Res. Commun.* 177, 867-873
  32. Preissner, K. T., and Jenne, D. (1991) *Thromb. Haemostasis* 66, 123-132
  33. Bittorf, S. V., Williams, E. C., and Mosher, D. F. (1993) *J. Biol. Chem.* 268, 24838-24846
  34. Tomasini, B. R., and Mosher, D. F. (1991) *Prog. Hemostasis Thromb.* 10, 269-305
  35. Altieri, D. C. (1991) *J. Immunol.* 147, 1891-1898
  36. Dransfield, I., Cabanas, C., Craig, A., and Hogg, N. (1992) *J. Cell Biol.* 116, 219-226
  37. Edwards, J. C., Hameed, H., and Campbell, G. (1988) *J. Cell Biol.* 89, 507
  38. Cox, D., Aoki, T., Seki, J., Motoyama, Y., and Yoshida, K. (1994) *Med. Res. Rev.* 14, 195-228
  39. Bednarczyk, J. L., and McIntyre, B. W. (1992) *J. Cell Biol.* 116, 499-509
  40. Campanero, M. R., Pulido, R., Ursa, M. A., Rodriguez-Moya, M., de Landazuri, M. O., and Sanchez-Madrid, F. (1990) *J. Cell Biol.* 110, 2157-2165
  41. Neugebauer, K. M., and Reichardt, L. F. (1991) *Nature* 350, 68-71
  42. O'Toole, T. E., Loftus, J. C., Du, X., Glass, A. A., Ruggeri, Z. M., Shattil, S. J., Plow, E. F., and Ginsberg, M. H. (1990) *Cell Regul.* 1, 883-893
  43. Zimolo, Z., Wesolowski, G., Tanaka, H., Hyman, J. L., Hoyer, J. R., and Rodan, G. A. (1994) *Am. J. Physiol.* 266, C376-C381
  44. Nesbitt, S., Nesbitt, A., Helfrich, M., and Horton, M. (1993) *J. Biol. Chem.* 268, 16737-16745
  45. Clover, J., Dodds, R. A., and Gowen, M. (1992) *J. Cell Sci.* 103, 267-271
  46. Saito, T., Albelda, S. M., and Brighton, C. T. (1994) *J. Ortho. Res.* 12, 384-394
  47. Hughes, D. E., Salter, D. M., Dedhar, S., and Simpson, R. (1993) *J. Bone Miner. Res.* 8, 527-533
  48. Holly, S., Sago, K., Martin, J., Chappel, J., Teitelbaum, S. L., Cao, X., and Ross, F. P. (1993) *J. Bone Miner. Res.* 8, S136
  49. Sago, K., Ross, F. P., Martin, J., Li, C-F., Chappel, J., Mimura, H., Reichardt, L. F., Venstrom, K., Teitelbaum, S. L., and Cao, X. (1993) *J. Bone Miner. Res.* 8, S121
  50. Reidy, M. A., Fingerle, J., and Majesky, M. W. (1988) in *Hyperlipidaemia and Atherosclerosis* (Suckling, K. E., and Groot, P. H. E., eds) pp. 149-164, Academic Press, London
  51. Giachelli, C. M., Bae, N., Almeida, M., Denhardt, D. T., Alpers, C. E., and Schwartz, S. M. (1993) *J. Clin. Invest.* 92, 1686-1689
  52. Yue, T. L., McKenna, P. J., Ohlstein, E. H., Farach-Carson, M. C., Butler, W. T., Johanson, K., McDevitt, P., Feuerstein, G. Z., and Stadel, J. M. (1994) *Exp. Cell Res.* 214, 459-464
  53. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* 51, 660-672

# Osteopontin-Induced, Integrin-Dependent Migration of Human Mammary Epithelial Cells Involves Activation of the Hepatocyte Growth Factor Receptor (Met)

Alan B. Tuck,<sup>1,2,3,4\*</sup> Bruce E. Elliott,<sup>5</sup> Charulata Hota,<sup>1,2,3,4</sup> Eric Tremblay,<sup>5</sup> and Ann F. Chambers<sup>1,2,4</sup>

<sup>1</sup>Department of Pathology, University of Western Ontario, London, Ontario, Canada N6A 5C1

<sup>2</sup>Department of Oncology, University of Western Ontario, London, Ontario, Canada N6A 5C1

<sup>3</sup>London Health Sciences Centre, London, Ontario, Canada N6A 4G5

<sup>4</sup>London Regional Cancer Centre, London, Ontario, Canada N6A 4L6

<sup>5</sup>Cancer Research Labs, Queen's University, Kingston, Ontario, Canada K7L 3N6

**Abstract** Osteopontin (OPN) is a secreted glycoposphoprotein which induces migration of mammary carcinoma cells, and has been implicated in the malignancy of breast carcinoma. Hepatocyte growth factor (HGF) induces cell migration of several mammary epithelial cell (MEC) lines, via activation of its cognate receptor (Met). This study examines the mechanism of OPN-induced MEC migration, in terms of the cell surface integrins involved and induction of the HGF/Met pathway. Three different MEC cell lines were used, representing different stages of tumor progression: 21PT, non-tumorigenic; 21NT, tumorigenic; non-metastatic; and MDA-MB-435, tumorigenic, highly metastatic. Human recombinant OPN was found to induce the migration of all three lines. OPN-induced migration of 21PT and 21NT cells was  $\alpha\text{v}\beta 5$  and  $\beta 1$ -integrin dependent, and  $\alpha\text{v}\beta 3$ -independent, while that of MDA-MB-435 cells was  $\alpha\text{v}\beta 3$ -dependent. HGF also induced migration of all three cell lines, and a synergistic response was seen to HGF and OPN together. The increased migration response to OPN was found to be associated with an initial increase in Met kinase activity (within 30 min), followed by an increase in Met mRNA and protein expression. OPN-induced cell migration is thus mediated by different cell surface integrins in MEC lines representing different stages of progression, and involves activation of the HGF receptor, Met. *J. Cell. Biochem.* 78:465–475, 2000. © 2000 Wiley-Liss, Inc.

**Key words:** osteopontin (OPN); cell migration; integrin; hepatocyte growth factor (HGF); Met; mammary epithelial cells; breast cancer

Growth, migration, and differentiation of epithelial cells are known to be dependent upon integrin-mediated adhesion to extracellular matrix components [reviews in Assoian, 1997; Bissell, 1999; Gumbiner, 1996]. Similarly, these same cellular processes are also known to

be influenced by a number of different growth factor pathways [reviews in Seedorf, 1995; Vande Woude et al., 1997; Heldin, 1998; Birchmeier, 1998]. Several recent studies have addressed the possibility of interactions between integrin and growth factor mediated pathways, with evidence emerging for both growth factor control of cell adhesion events [van der Voort et al., 1997; Weimar et al., 1997; Trusolino et al., 1998; Weimar et al., 1998], and conversely, for integrin-mediated cell adhesion phenomena influencing sensitivity to certain growth factors [Miyamoto et al., 1996; Brooks et al., 1997]. However, the mechanism and biological relevance of these growth factor-integrin interactions are not yet clear.

Our group has particular interest in the role of the secreted glycoposphoprotein OPN in the malignancy of breast cancer. We have found that OPN can induce cell migration and

Grant sponsor: Canadian Breast Cancer Research Initiative; Grant number: 8426; Grant sponsor: U.S. Army Medical Research and Materials Command; Grant number: DAMD17-96-1-6251; Grant sponsor: U.S. Army Breast Cancer Research Program; Grant number: DAMD17-96-1-6075.

The content of this article does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

\*Correspondence to: Alan B. Tuck, MD, PhD, Department of Pathology, London Health Sciences Centre, Victoria Campus, 375 South St., London, Ontario N6A 4G5, Canada. E-mail: atuck@julian.uwo.ca

Received 6 December 1999; Accepted 20 March 2000

Print compilation © 2000 Wiley-Liss, Inc.

invasiveness of cultured mammary epithelial cells (MECs) [Xuan et al., 1994; Xuan et al., 1995; Tuck et al., 1999], that it may be secreted in greater quantity by MECs of greater degree of malignancy [Tuck et al., 1999], and that higher levels (tumor cell or plasma levels respectively) are associated with poorer prognosis in patients with either lymph node negative or metastatic breast cancer [Tuck et al., 1998; Singhal et al., 1997]. Evidence from us [Xuan et al., 1994, 1995; Tuck et al., 1999], and others [Senger et al., 1996], has indicated that OPN-induced cell movement is a directed, RGD-dependent response, although CD44-mediated phenomena may also be involved [Weber et al., 1996; Bourguignon et al., 1998, 1999; Katagiri et al., 1999; Tuck et al., unpublished observations]. Cell adhesion studies have shown for a variety of cell types that the major cell surface integrins involved in OPN binding include  $\alpha\beta1$ ,  $\alpha\beta3$ , and  $\alpha\beta5$  [Hu et al., 1995; Liaw et al., 1995].

Given this information, along with the abundant evidence for the importance of HGF/Met in cell motility of MECs [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al., 1998], and in the malignancy of breast cancer [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Beviglia et al., 1997; Ghossoub et al., 1998], we set out to examine the nature of OPN-induced cell migration, with respect to the involvement of cell surface integrins known to bind OPN, and possible interactions with the HGF/Met pathway. We have made use of three MEC lines, of differing malignancy: 21PT, non-tumorigenic; 21NT, tumorigenic, non-metastatic [Band et al., 1990]; and MDA-MB-435: tumorigenic, highly metastatic [Price et al., 1990]. We have assessed these cells for migratory responsiveness to OPN, alone and in combination with HGF. Having found evidence for a synergistic relationship between OPN and HGF in inducing cell migration, we proceeded to characterize the cell surface integrins involved, using blocking antibodies to  $\alpha\beta5$ ,  $\beta1$ , or  $\alpha\beta3$  integrins. OPN-treated cells were then examined in time course experiments for induction of Met kinase activity and tyrosine phosphorylation, and for levels of HGF and Met mRNA and protein. Incubation with OPN was found to result in rapid activation of Met (all three cell lines), followed by an increase in Met RNA (all three cell lines) and protein (21PT and 21NT).

This work thus provides evidence that MEC cell lines representative of different stages of progression make use of different cell surface integrins in the migration response to OPN, and that this OPN-induced cell migration may be mediated at least in part by activation of Met.

## METHODS

### Cell Lines and Culture

The 21T series cell lines (21PT, 21NT) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute) [Band et al., 1990]. These cells were maintained in culture in  $\alpha$ -MEM supplemented with 10% FCS, 2 mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY), insulin (1  $\mu$ g/ml), epidermal growth factor (EGF; 12.5 ng/ml), hydrocortisone (2.8  $\mu$ M), 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50  $\mu$ g/ml gentamycin (all from Sigma;  $\alpha$ HE medium). MDA-MB-435 cells [Price et al., 1990] were obtained as a kind gift of Dr. Janet Price (MD Anderson Cancer Center, Houston, TX), and were grown in  $\alpha$ -MEM with 10% FCS (both from GIBCO-BRL/Life Technologies).

### Cell Migration

Cell migration assays were performed essentially as described previously [Xuan et al., 1995], using 24-well transwell chambers with polycarbonate filters of 8  $\mu$ m pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6  $\mu$ g/filter and air dried. The gelatin was rehydrated with 100  $\mu$ l of serum-free  $\alpha$ HE medium at room temperature for 90 min. Lower wells contained 800  $\mu$ l of  $\alpha$ HE plus 0.1% BSA, with or without OPN, HGF, and/or blocking antibodies (as specified in Figs. 1 and 2). Human OPN (50  $\mu$ g/ml) used was the full length human recombinant GST-OPN (hrOPN), as previously described [Xuan et al., 1994]. Previous control experiments have shown that the GST portion alone has no influence on migration of these cells. Human HGF (20 ng/ml) was obtained from Collaborative Biomedical Products (Becton-Dickinson, Bedford, MA). Blocking anti-integrin antibodies included anti- $\alpha\beta3$  (Cedarlane, Hornby, ON), anti- $\alpha\beta5$  (GIBCO-BRL), and anti- $\beta1$  (GIBCO-BRL), all used at saturating concentrations as determined by preliminary titration experiments. Cells



( $5 \times 10^4$ ) were added to each upper well in  $\alpha$ HE medium with 0.1% BSA and incubated for 5 h at 37°C. At the end of the incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with glutaraldehyde and stained with hematoxylin. Cells that had not migrated and were attached to the

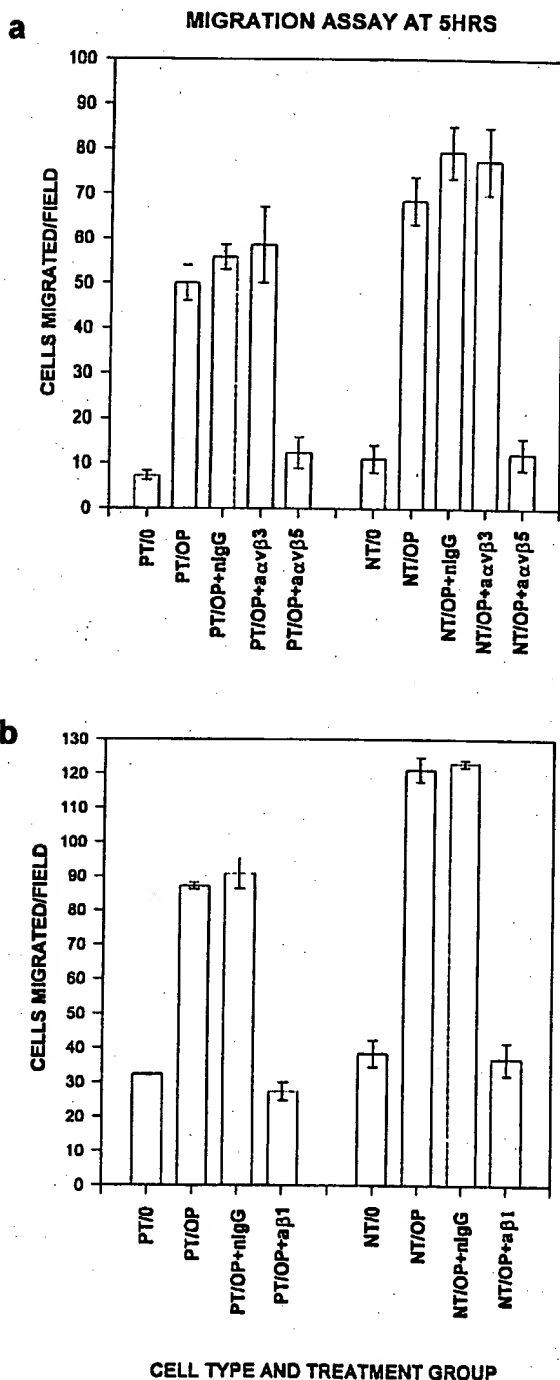
upper surface of the filters were removed from the filters with a cotton swab. The lower surfaces of the filters were examined microscopically under 100 $\times$  magnification and representative areas were counted to determine the number of cells that had migrated through the filters. Control experiments were also performed in which blocking antibody in the lower chamber was replaced by non-immune mouse IgG (Cedarlane) at comparable concentration.

All cell migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using Student's *t*-test, with SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

#### Immunoprecipitation and Western Blotting for Met and Phosphotyrosine

Cells in monolayer were grown to 85–90% confluence, serum starved overnight, and incubated in serum-free medium either with or without human OPN (50  $\mu$ g/ml) or HGF (20 ng/ml) for the times specified. Cells were then rinsed with cold PBS, and lysed in lysis buffer containing 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF, 2 mM EGTA, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal protein amounts of each lysate were immunoprecipitated with rabbit anti-human Met polyclonal antibody at 4°C for 2 h.

Immunoprecipitates were collected on protein A-Sepharose (Amersham-Pharmacia Bio-



**Fig. 1.** a: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is  $\alpha$ v $\beta$ 5, not  $\alpha$ v $\beta$ 3, integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50  $\mu$ g/ml hrOPN only (OP); 50  $\mu$ g/ml hrOPN with 15  $\mu$ g/ml non-specific mouse IgG (OP+nlG); 50  $\mu$ g/ml hrOPN with 30  $\mu$ g/ml anti- $\alpha$ v $\beta$ 3 integrin blocking antibody (OP+ $\alpha$ v $\beta$ 3); or 50  $\mu$ g/ml hrOPN with 15  $\mu$ g/ml anti- $\alpha$ v $\beta$ 5 integrin blocking antibody (OP+ $\alpha$ v $\beta$ 5). b: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is  $\beta$ 1 integrin-dependent. Lower chamber conditions were as follows: 0.1% BSA only (0); 50  $\mu$ g/ml hrOPN only (OP); 50  $\mu$ g/ml hrOPN with 15  $\mu$ g/ml non-specific mouse IgG (OP+nlG); or 50  $\mu$ g/ml hrOPN with 15  $\mu$ g/ml anti- $\beta$ 1 integrin blocking antibody (OP+ $\alpha$ 1). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.



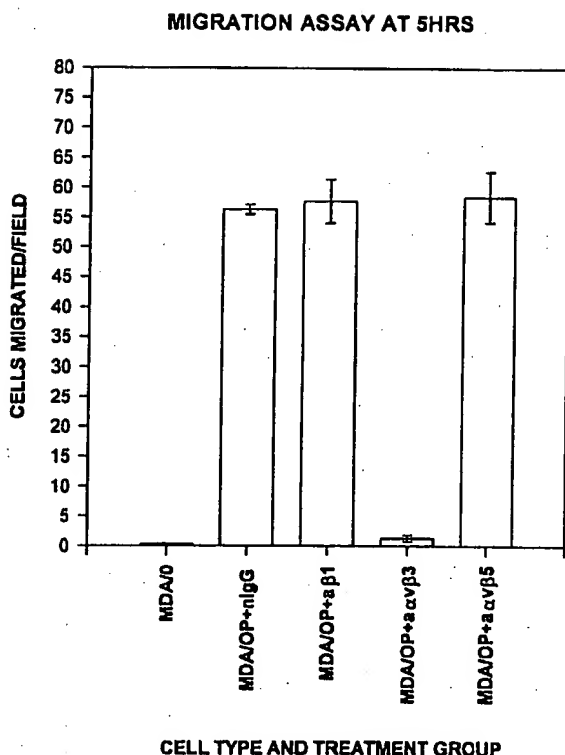


Fig. 2. OPN-induced migration of MDA-MB-435 (MDA) cells is  $\alpha\beta 3$ , not  $\alpha\beta 5$  or  $\beta 1$  integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50  $\mu\text{g/ml}$  hrOPN only (OP); 50  $\mu\text{g/ml}$  hrOPN with 25  $\mu\text{g/ml}$  non-specific mouse IgG (OP+nlG); 50  $\mu\text{g/ml}$  OPN with 15  $\mu\text{g/ml}$  anti- $\beta 1$  integrin blocking antibody (OP+ $\alpha\beta 1$ ); 50  $\mu\text{g/ml}$  hrOPN with 25  $\mu\text{g/ml}$  anti- $\alpha\beta 3$  integrin blocking antibody (OP+ $\alpha\beta 3$ ); or 50  $\mu\text{g/ml}$  hrOPN with 15  $\mu\text{g/ml}$  anti- $\alpha\beta 5$  integrin blocking antibody (OP+ $\alpha\beta 5$ ). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

tech, Baie d'Urfe, Quebec, Canada), washed three times with lysis buffer, separated by 7% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skim milk, or 1% BSA, in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with either mouse anti-human Met (DL-21 clone, Upstate Biotechnology Inc., Lake Placid, NY) or anti-phosphotyrosine antibody (PY20 clone, Transduction Labs, Lexington, KY). The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-mouse antibody (Amersham-Pharmacia Biotech) for 15 min, and washed three times with TBST for

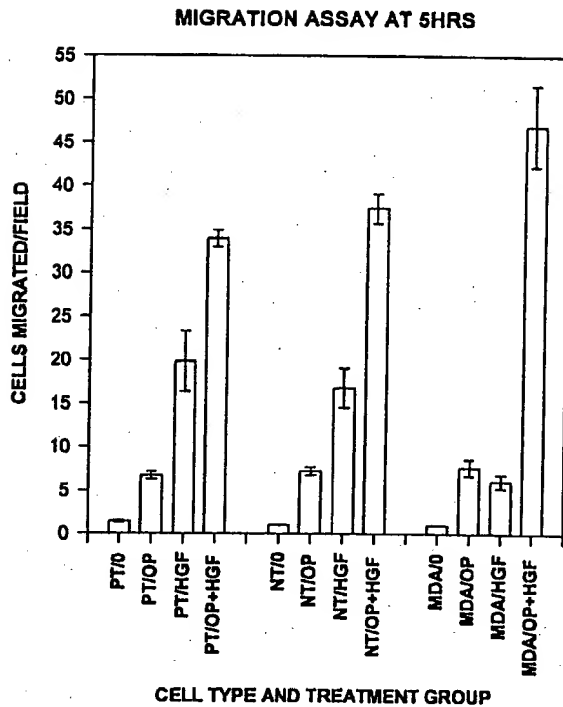
10 min each. Immune complexes were detected using ECL (Mandel/NEN, Guelph, ON).

#### In Vitro Met Kinase Assay

Cell cultures incubated in the presence or absence of hrOPN (50  $\mu\text{g/ml}$ ) or HGF (20 ng/ml) were rinsed with cold PBS, lysed, and immunoprecipitated as above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase buffer (20 mM PIPES, pH 7.0, 10 mM  $\text{MnCl}_2$ , 10  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ ). In vitro Met kinase activity was determined by incubating immunoprecipitates with 20  $\mu\text{l}$  of kinase buffer containing 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP at 30°C for 10 min. The reaction was stopped by addition of 2 $\times$  SDS sample buffer containing 5%  $\beta$ -mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45°C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at 80°C under a vacuum. Autoradiograms were produced and quantitated using a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

#### Analysis of Met and HGF mRNA Levels

Cell cultures (85–90% confluent) were incubated in serum-free medium for the specified times in the presence or absence of 50  $\mu\text{g/ml}$  hrOPN. Cells were harvested by gentle trypsinization, pelleted, and mechanically homogenized (Polytron PT 1200, Brinkman Instruments [Canada] Ltd., Mississauga, ON). RNA was extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON), according to the protocol supplied by the manufacturer. RNA (10  $\mu\text{g/lane}$ ) was run on a 1.1% agarose gel with 6.8% formaldehyde, and capillary-transferred to GeneScreen Plus filters (DuPont Canada Inc., Mississauga, ON). Blots were probed with denatured, oligolabeled [ $\alpha$ - $^{32}\text{P}$ ]-dCTP cDNA probes (labeled using a kit provided by Pharmacia), according to the procedures provided by the manufacturers, and as previously described [Tuck et al., 1990, 1991]. cDNA probes were as follows: hepatocyte growth factor (HGF)—540 bp BamHI-XhoI fragment of human HGF cDNA [Nakamura et al., 1989]; Met/HGF receptor (HGFR)—800 bp EcoRI-EcoRV fragment of the human *met* cDNA [Park et al., 1987]; 18S rRNA (18S)—from p100D9.

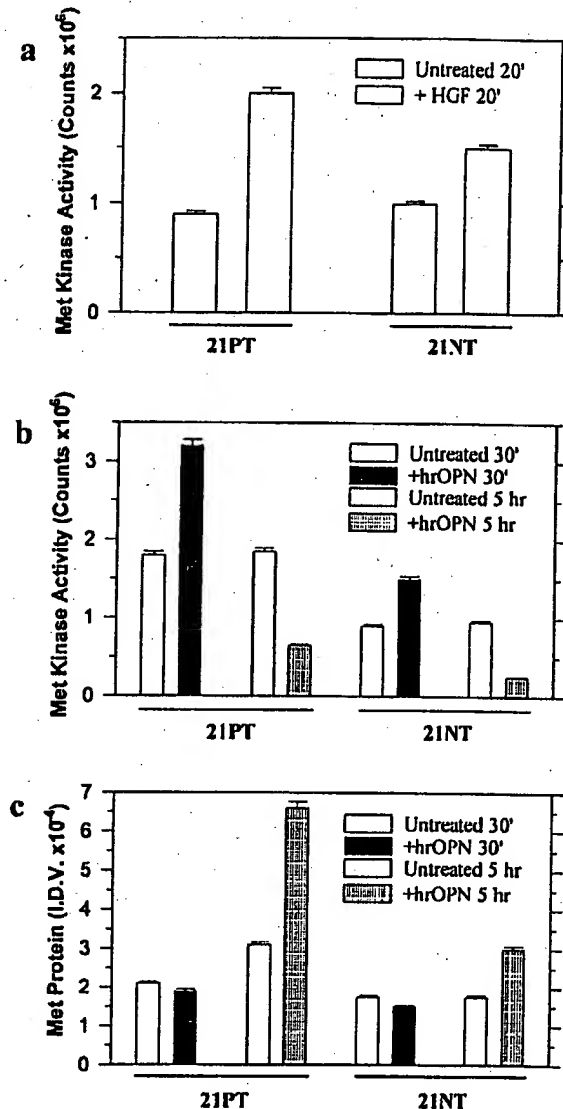


**Fig. 3.** Synergistic effect of OPN and HGF on migration of 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Migration assays were performed as described in Materials and Methods. Contents of the lower chamber consisted of either: medium ( $\alpha$ H, no EGF) without HGF or hrOPN (O); medium with 50  $\mu$ g/ml hrOPN (OP); medium with 10 ng/ml HGF (HGF); or medium with 50  $\mu$ g/ml hrOPN and 10 ng/ml HGF (OP+HGF). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

## RESULTS

### OPN-Induced Migration of 21PT and 21NT Cells Involves Different Cell Surface Integrins Than for MDA-MB-435 Cells

Cell migration of 21PT, 21NT, and MDA-MB-435 cells was found to occur in response to hrOPN at a level comparable to that determined previously [Tuck et al., 1999]. Blocking experiments were performed using saturating concentrations of anti-integrin antibodies in the lower chamber of transwells, as described in Methods. For 21PT and 21NT cells, complete blocking of OPN-induced cell migration (to baseline levels) was obtained with the anti- $\alpha$ v $\beta$ 5 and  $\beta$ 1 integrin antibodies (Fig. 1a,b;  $P < 0.002$  for all, Student's *t*-test). In contrast, non-immune mouse IgG did not block migration of either cell line. Saturating concentrations (30  $\mu$ g/ml) of anti- $\alpha$ v $\beta$ 3 integrin antibody had no detectable effect on migration of either 21PT or 21NT cells (Fig. 1a).



**Fig. 4.** HGF (a) and OPN (b)-induced increase in total cellular Met kinase activity of 21PT and 21NT cells. OPN (c)-induced increase in total Met protein of 21PT and 21NT cells. a,b: Cells were incubated  $\pm$ 20 ng/ml HGF or 50  $\mu$ g/ml hrOPN for the times indicated, and cells lysates were prepared. Equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG, and in vitro Met kinase activity was determined as described in Materials and Methods. Quantitation was done using a Phosphorimager. Total Met kinase activity is expressed in cpm/sample. c: Met protein was quantitated by immunoprecipitation with rabbit polyclonal anti-Met antibody, followed by 7% SDS-PAGE and Western blotting as described in Materials and Methods. Total Met protein was quantitated by densitometry and is expressed in integrated density value units. Error bars represent an average standard deviation of 2.5%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

In contrast to results with 21PT and 21NT cells, anti- $\alpha\text{v}\beta 5$  and  $\beta 1$  integrin antibodies showed no blocking effect on OPN-induced cell migration of MDA-MB-435 cells, when used at the same high concentrations shown to effect complete blocking of 21PT and 21NT responsiveness (15  $\mu\text{g}/\text{ml}$  of either anti-integrin antibody; Fig. 2). On the other hand, OPN-induced migration of MDA-MB-435 cells was completely blocked by anti- $\alpha\text{v}\beta 3$  integrin antibody, at a concentration (25  $\mu\text{g}/\text{ml}$ ) lower than that which still had no effect on migration of 21PT or 21NT (30  $\mu\text{g}/\text{ml}$ ; cf. Fig. 1;  $P = 0.0008$ , Student's *t*-test).

The OPN-induced migration of the metastatic cell line of this series—MDA-MB-435, thus was found to be  $\alpha\text{v}\beta 3$  integrin-dependent, whereas that of non-metastatic 21NT and 21PT cells was  $\alpha\text{v}\beta 5$  and  $\beta 1$ -dependent,  $\alpha\text{v}\beta 3$ -independent.

#### HGF-Induced Cell Migration and Synergistic Effect With OPN

As was found for response to OPN, all three cell lines (21PT, 21NT, MDA-MB-435) showed increased cell migration in response to human recombinant HGF alone (Fig. 3). Combining both HGF and osteopontin in the lower chamber resulted in a degree of cell migration for all three cell lines that was significantly greater than the sum of the isolated HGF and OPN responses (i.e., synergistic; Fig. 3;  $P < 0.02$  for all, Student's *t*-test).

#### Induction of Met (HGFR) Activity by HGF and OPN

Treatment of 21PT and 21NT cells with either HGF or OPN (Fig. 4a,b) resulted in rapid activation of total Met kinase activity in both instances (after 20 min of HGF stimulation, 30 min of OPN stimulation). For both 21PT and 21NT cells, the increase in total Met kinase activity with OPN treatment (at 30 min) was followed by an increase in Met protein level at 5 h of incubation with OPN (Fig. 4c).

Treatment of MDA-MB-435 cells with HGF or OPN (Fig. 5a) resulted in an increase in specific Met kinase activity after 20 (for HGF) to 30 (for OPN) min of incubation, which was associated with an increased tyrosine phosphorylation of Met as well (Fig. 5b). In contrast with 21PT and 21NT cells, we have not been able to detect an OPN-induced increase in total

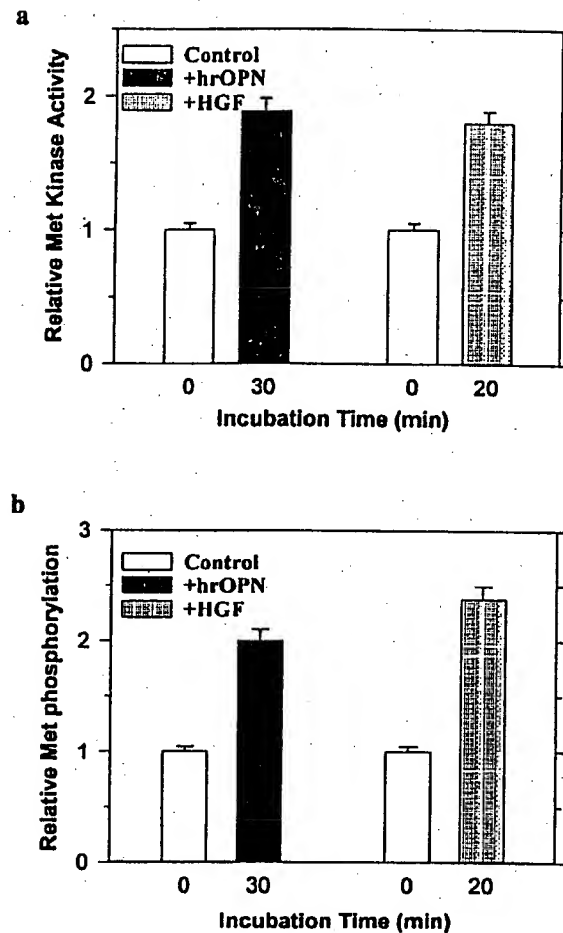


Fig. 5. Induction of specific Met kinase activity (a) and Met tyrosine phosphorylation (b) of MDA-MB-435 cells by HGF and OPN. MDA-MB-435 cells were incubated  $\pm 50 \mu\text{g}/\text{ml}$  hrOPN or 20 ng/ml HGF for the times indicated. Cell lysates were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG. As levels of total Met protein were higher and fluctuated more in MDA-MB-435 cells than in 21PT and 21NT, activation of Met protein in MDA-MB-435 was more appropriately expressed as Relative Met kinase activity (a) and tyrosine phosphorylation (b). a: In vitro Met kinase activity was assayed as described in Materials and Methods. Relative Met kinase activity, normalized to total Met protein, was quantitated using a Phosphorimager. b: Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with 1% BSA in TBST, and probed with anti-phosphotyrosine antibody. Detection was performed with HRP-labeled anti-mouse antibody and ECL. Relative Met tyrosine-phosphorylation normalized to total Met protein was quantitated by densitometry. Error bars represent an average standard deviation of 5.0%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

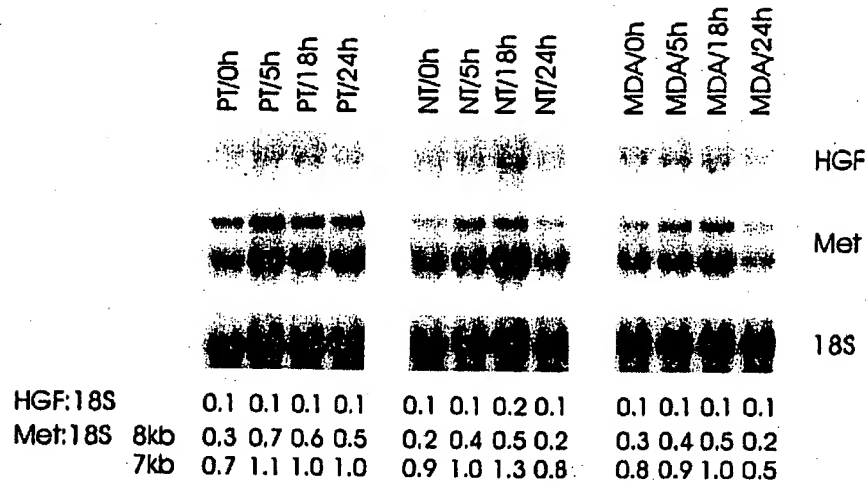


Fig. 6. Time course showing effect of OPN on expression of HGF and Met mRNA by 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Near-confluent (85–90%) cell cultures were incubated in serum-free medium with 50  $\mu$ g/ml OPN for 0, 5, 18, or 24 h. Total RNA (10  $\mu$ g/lane) was analysed by Northern blotting for expression of HGF (6.0 kb) or Met (HGFR; 8.0 kb [full-length transcript, upper band], 7.0 kb [lower band]). RNA loading and integrity were verified by assessment of 18S rRNA (2.1 kb). Level of HGF and Met (both 8kb and 7kb transcripts) are shown in relation to 18S rRNA, expressed as the ratio of densitometry values for the respective bands (HGF:18S, Met:18S).

Met protein levels in MDA-MB-435 cells, although basal level of Met protein expression in MDA-MB-435 is higher than in 21PT or 21NT (data not shown).

#### Time Course Showing Effect of OPN on Expression of HGF and Met mRNA

21PT, 21NT, and MDA-MB-435 cells treated with OPN (50  $\mu$ g/ml) for 0–24 h (Fig. 6), all showed low basal levels of HGF mRNA. Only slight increase in HGF mRNA was detected for 21PT and 21NT, with no appreciable increase for MDA-MB-435 cells (by 18–24 h). In contrast, levels of Met RNA were found to significantly increase in all three cell lines between 5 and 18 h of OPN exposure, falling off by 24 h. Thus, although little change in HGF mRNA was seen after up to 24 h of OPN exposure, significant induction of Met mRNA was seen for all three cell lines.

#### DISCUSSION

OPN has been implicated in the malignancy of breast cancer in a number of recent studies [e.g., Oates et al., 1996; Singhal et al., 1997; Sung et al., 1998; Tuck et al., 1998, 1999]. It has been shown to be involved in cell adhesion of MECs, and can also induce cell migration in an RGD-dependent fashion [Xuan et al., 1994, 1995; Senger et al., 1996]. The HGF/Met path-

way has also been associated with breast cancer malignancy [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Beviglia et al., 1997; Ghoussoub et al., 1998], and is a potent inducer of MEC motility [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al., 1998]. Here we examine the nature of the integrin response to OPN, in order to establish the specific cell surface integrins involved. We also show that OPN-induced cell migration involves activation of the HGF receptor in a synergistic fashion with HGF, consistent with cross-talk between integrin and growth factor mediated pathways.

Our discovery that, in a series of breast epithelial cells of differing degrees of malignancy, different cell surface integrins may couple with Met in inducing cell migration is a novel finding. The metastatic member of the series studied, MDA-MB-435 cells, showed the most marked synergy between OPN and HGF in the migration response, and migrated in an  $\alpha$ v $\beta$ 3, not  $\alpha$ v $\beta$ 5 or  $\beta$ 1-dependent fashion. In contrast, the non-metastatic cell lines, 21PT and 21NT, migrated in an  $\alpha$ v $\beta$ 5 and  $\beta$ 1 dependent,  $\alpha$ v $\beta$ 3-independent fashion. In support of this finding is the work of Wong et al. [1998], who reported that MDA-MB-435 cells express  $\alpha$ v $\beta$ 3 integrin, while less malignant MDA-MB-231 and MCF-7 cells do not (although they all express  $\alpha$ v $\beta$ 5 and

β1). Similarly, van der Pluijm et al. [1997] reported higher expression of αvβ3 in more malignant members of a series of breast carcinoma cell lines. A specific association between αvβ3 expression and breast cancer metastasis has also been reported by Liapis et al. [1996], who detected αvβ3 integrin expression in 100% of breast carcinomas that had metastasized to bone.

This difference in integrin utilization of cells at different stages of progression could affect malignancy in a number of different ways. For example, αvβ3 may be necessary for specific adhesion events vital to invasion and metastasis; a breast cancer cell initially expressing αvβ1 or αvβ5 may require activation of β3 in order to complete that step of the metastatic cascade. Evidence in favor of this scenario (at least in the case of melanoma) comes from the work of Nip et al. [1992], who showed that binding of metastatic cells to lymph node matrix depends on αvβ3 interactions. Alternatively, different integrins may be coupled to different signal transduction pathways, with αvβ3 specifically required for activation of a particular set of genes important in aspects of invasion and metastasis. Ligation of αvβ3 for example, has been shown to induce MMP-2 expression and invasion of melanoma cells [Seftor et al., 1992; Bafetti et al., 1998]. Whether or not different integrins couple to Shc can influence activation of transcription from the Fos serum response element (SRE), affecting responsiveness to growth factors [Wary et al., 1996]. In the case of coupling with the HGF/Met pathway, our work suggests that OPN-induced cell migration via either non-αvβ3 integrins (21PT, 21NT) or αvβ3 integrin (MDA-MB-435) is associated with Met activation, but that the synergistic effect on cell migration is much more pronounced in the cells (MDA-MB-435) expressing αvβ3.

We also found that OPN-induced migration of all three cell lines involves activation of the HGF receptor, Met, with an initial increase in Met activity followed by an increase in Met RNA expression. The kinetics of this effect differ slightly for MDA-MB-435 vs. the 21T series cells. For 21PT and 21NT, a detectable increase in Met protein was also found. Although a similar increase in Met protein levels of MDA-MB-435 cells did not occur, the basal level of Met protein in these

cells is quite high, and as specific activity of Met is substantially increased with OPN induction, it is possible that Met turnover is such that protein levels do not further accumulate as they do for the 21T series cells. Regardless, Met is activated by OPN in all three cell lines, and this is associated with increased cell migration. In contrast, HGF mRNA levels were low in all three cell lines, and showed little or no change with OPN treatment. Furthermore, we have not detected increased HGF activity in conditioned media of OPN-treated 21T series or MDA-MB-435 cells (data not shown). Activation of Met by OPN is thus likely due to either an increased sensitivity to trace amounts of ligand present, or to ligand-independent activation. Ligand-independent activation of Met by cellular adhesion has been previously reported for melanoma cells, although the cell surface adhesion receptors involved were not examined [Wang et al., 1996]. Furthermore, integrin binding has been shown to be essential for growth factor (EGF, PDGF, bFGF, IGF-1) induced signal transduction and cell migration [Miyamoto et al., 1996; Brooks et al., 1997]. Reciprocally, HGF can activate cell surface integrins and hence cellular adhesion (and motility) [van der Voort et al., 1997; Weimar et al., 1997, 1998; Trusolino et al., 1998]. Thus, a two-way interaction between integrin and growth factor-mediated pathways likely occurs in the induction of cellular responses such as cell migration.

Multiple points of interaction between signal transduction pathways activated by integrins vs. growth factors have been identified [reviewed in Sastry and Horowitz, 1996; Giancotti, 1997; Swartz, 1997]. Cell attachment can enhance autophosphorylation of growth factor receptors (EGFR, PDGFR, and now Met) in response to their cognate ligands. Integrin binding also has been found to activate phospholipase C (and hence protein kinase C), Raf, and/or MEK in the MAP kinase pathway, and PI-3 kinase in the PI-3K/Rac pathway. All of these pathways are also influenced by growth factors, although the synergistic relationship reported here would suggest that growth factor receptors and integrins may act at different points in the pathway. For example, it has been shown that fibronectin binding to cell surface integrin activates synthesis and supply of phosphatidylinositol 4,5 biphosphate, whereas PDGF receptor controls the activity of phospho-

lipase C [McNamee et al., 1992]. The physical association of integrins and growth factor receptors at the focal adhesion complex (FAC) [Plopper et al., 1995] provides a mechanism by which such cross-talk would be facilitated. Finally, interactions between growth factor and integrin pathways could occur at the level of differential effects on members of the FAC itself [Schlaepfer and Hunter, 1998].

In the case of signal transduction initiated by OPN, ligation of  $\alpha v \beta 3$  by OPN activates PI-3 kinase in osteoclasts [Hruska et al., 1995]. In an osteoblastic cell line (UMR 106-6), OPN triggers the autophosphorylation of focal adhesion kinase (FAK) [Liu et al., 1997]. In *ras*-transformed fibroblasts, OPN can induce tyrosine phosphorylation of a number of different FAC associated proteins [Lopez et al., 1995]. HGF activation of its receptor, Met, can also stimulate phosphorylation of FAK in some cells, perhaps via  $pp60^{src}$  [Chen et al., 1998]. We have shown in this report that OPN is also capable of activating Met. Thus, although our understanding of the signal transduction pathways induced by OPN is yet in early stages, multiple points of potential interaction between the integrin and growth factor receptor pathways involved are already beginning to emerge.

The interactions between different integrin pathways induced by OPN and the HGF/Met growth factor pathway not only helps conceptually in understanding the clinical associations we have observed between OPN, HGF and malignancy, but also provides clues to regulatory processes vital to tumor aggressiveness—prime targets for treatment strategies based on blocking these processes.

#### ACKNOWLEDGMENTS

Dr. Tuck is supported by a "Career Development" Award from the U.S. Army Breast Cancer Research Program.

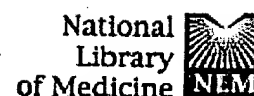
#### REFERENCES

- Assoian RK. 1997. Anchorage-dependent cell cycle progression. *J Cell Biol* 136:1–4.
- Bafetti LM, Young TN, Itoh Y, Stack MS. 1998. Intact vitronectin induces matrix metalloproteinase-2 and tissue inhibitor of metalloproteinases-2 expression and enhanced cellular invasion by melanoma cells. *J Biol Chem* 273:143–149.
- Band V, Zajchowski D, Swishhelm K, Trask D, Kulesa V, Cohen C, Connolly J, Sager R. 1990. Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res* 50:7351–7357.
- Beviglia L, Matsumoto K, Lin CS, Ziober BL, Kramer RH. 1997. Expression of the c-Met/HGF receptor in human breast carcinoma: Correlation with tumor progression. *Int. J. Cancer* 74:301–309.
- Bhargava M, Joseph A, Knesel J, Halaban R, Li Y, Pang S, Goldberg I, Setter E, Donovan MA, Zarnegar R, Michalopoulos GA, Nakamura T, Faletto D, Rosen EM. 1992. Scatter factor and hepatocyte growth factor: Activities, properties, and mechanism. *Cell Growth Differ* 3:11–20.
- Birchmeier C, Gherardi E. 1998. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol* 8:404–410.
- Bissell MJ, Weaver VM, Lelievre SA, Wang F, Petersen OW, Schmeichel KL. 1999. Tissue structure, nuclear organization, and gene expression in normal and malignant breast. *Cancer Res* 59:1757–1763.
- Bourguignon LY, Gunja-Smith Z, Iida N, Zhu HB, Young LJ, Muller WJ, Cardiff RD. 1998. CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J Cell Physiol* 176:206–215.
- Bourguignon LY, Zhu H, Shao L, Zhu D, Chen YW. 1999. Rho-kinase (ROK) promotes CD44v(3,8-10)-ankyrin interaction and tumor cell migration in metastatic breast cancer cells. *Cell Motil Cytoskeleton* 43:269–287.
- Brooks PC, Klemke RL, Schön S, Lewis JM, Schwartz MA, Cheresch DA. 1997. Insulin-like growth factor receptor cooperates with integrin  $\alpha v \beta 5$  to promote tumor cell dissemination in vivo. *J Clin Invest* 99:1390–1398.
- Chen HC, Chan PC, Tang MJ, Cheng CH, Chang TJ. 1998. Tyrosine phosphorylation of focal adhesion kinase stimulated by hepatocyte growth factor leads to mitogen-activated protein kinase activation. *J Biol Chem* 273:25777–25782.
- Ghoussoub RA, Dillon DA, D'Aquila T, Rimm EB, Fearon ER, Rimm DL. 1998. Expression of c-met is a strong independent prognostic factor in breast carcinoma. *Cancer* 82:1513–1520.
- Giancotti FG. 1997. Integrin signaling: Specificity and control of cell survival and cell cycle progression. *Curr Opin Cell Biol* 9:691–700.
- Gumbiner BM. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84:345–357.
- Heldin CH, Ostman A, Ronnstrand L. 1998. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1378:F79–113.
- Hruska K, Rolnick F, Huskey M, Alvarez U, Cheresch D. 1995. Engagement of the osteoclast integrin  $\alpha v \beta 3$  by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. In: Denhardt DT, Butler WT, Chambers AF, Senger DR, editors. *Osteopontin—role in cell signaling and adhesion*. *Ann NY Acad Sci* 760:151–165.
- Hu DD, Lin EC, Kovach NL, Hoyer JR, Smith JW. 1995. A biochemical characterization of the binding of osteopontin to integrins  $\alpha v \beta 1$  and  $\alpha v \beta 5$ . *J Biol Chem* 270:26232–26238.
- Jin L, Fuchs A, Schnitt SJ, Yao Y, Joseph A, Lamszus K, Park M, Goldberg ID, Rosen EM. 1997. Expression of scatter factor and c-met receptor in benign and malignant breast tissue. *Cancer* 79:749–760.

- Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T. 1999. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. *Cancer Res* 59:219-226.
- Liapis H, Flath A, Kitazawa S. 1996. Integrin  $\alpha v \beta 3$  expression by bone-residing breast cancer metastases. *Diag Mol Pathol* 5:127-135.
- Liaw L, Skinner MP, Raines EW, Ross R, Cheresch DA, Schwartz SM, Giachelli CM. 1995. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. *J Clin Invest* 95:713-724.
- Liu Y-K, Uemura T, Nemoto A, Yabe T, Fuhii N, Ushida T, Tateishi T. 1997. Osteopontin involvement in integrin-mediated cell signaling and regulation of expression of alkaline phosphatase during early differentiation of UMR cells. *FEBS Lett* 420:112-116.
- Lopez CA, Davis RL, Mou K, Denhardt DT. 1995. Activation of a signal transduction pathway by osteopontin. In: Denhardt DT, Butler WT, Chambers AF, Senger DR, editors: *Osteopontin—role in cell signaling and adhesion*. Ann NY Acad Sci 760:324-326.
- McNamee HM, Ingber DE, Schwartz MA. 1992. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol breakdown. *J Cell Biol* 121:673-678.
- Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: Roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 135:1633-1642.
- Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. 1989. Molecular cloning and expression of hepatocyte growth factor. *Nature* 34:440-443.
- Nip J, Shibata H, Loskutoff DJ, Cheresch DA, Brodt P. 1992. Human melanoma cells derived from lymphatic metastases use integrin alpha v beta 3 to adhere to lymph node vitronectin. *J Clin Invest* 90:1406-1413.
- Oates AJ, Barraclough R, Rudland PS. 1996. The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. *Oncogene* 13:97-104.
- Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G. 1987. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. *Proc Natl Acad Sci USA* 84:6379-6383.
- Plopper G, McNamee HP, Dike LE, Bojanowski K, Ingber DE. 1995. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell* 6:1349-1365.
- Price JE, Polyzos A, Zhang RD, Daniels LM. 1990. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res* 50:717-721.
- Rahimi N, Hung W, Tremblay E, Saulnier R, Elliott B. 1998. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J Biol Chem* 273:33714-33721.
- Rosen EM, Knesel J, Goldberg ID, Jin L, Bhargava M, Joseph A, Zitnik R, Wines J, Kelley M, Rockwell S. 1994. Scatter factor modulates the metastatic phenotype of the EMT6 mouse mammary tumor. *Int J Cancer* 57:706-714.
- Sastry SK, Horowitz AF. 1996. Adhesion - growth factor interactions during differentiation: An integrated biological response. *Dev Biol* 180:455-467.
- Schlaepfer DD, Hunter T. 1998. Integrin signalling and tyrosine phosphorylation: Just the FAKs? *Trends Cell Biol* 8:151-157.
- Schwartz MA. 1997. Integrins, oncogenes, and anchorage independence. *J Cell Biol* 139:575-578.
- Seedorf K. 1995. Intracellular signaling by growth factors. *Metabolism* 44:24-32.
- Seftor RE, Seftor EA, Gehlsen KR, Statler-Stevenson WG, Brown, PD, Ruoslahti E, Hendrix MJ. 1992. Role of the alpha v beta 3 integrin in human melanoma cell invasion. *Proc Natl Acad Sci* 89:1557-1561.
- Senger DR, Perruzzi CA. 1996. Cell migration promoted by a potent GRGDS-containing thrombin-cleavage fragment of osteopontin. *Biochim Biophys Acta* 1314:13-24.
- Singhal H, Bautista DS, Tonkin KS, O'Malley FP, Tuck AB, Chambers AF, Harris JF. 1997. Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. *Clin Cancer Res* 3:605-611.
- Sung V, Gilles C, Murray A, Clarke R, Aaron AD, Azumi N, Thompson EW. 1998. The LCC15-MB human breast cancer cell line expresses osteopontin and exhibits an invasive and metastatic phenotype. *Exp Cell Res* 241:273-284.
- Trusolino L, Serini G, Cecchini G, Basati C, Ambesi-Impimato FS, Marchisio PC, de Filippi R. 1998. Growth factor-dependent activation of  $\alpha v \beta 3$  integrin in normal epithelial cells: Implications for tumor invasion. *J Cell Biol* 142:1145-1156.
- Tuck AB, Park M, Sterns EE, Boag A, Elliott BE. 1996. Coexpression of hepatocyte growth factor and receptor (met) in human breast carcinoma. *Am J Pathol* 148:225-232.
- Tuck AB, O'Malley FP, Singhal H, Harris JF, Tonkin KS, Kerkvliet N, Saad Z, Doig GS, Chambers AF. 1998. Osteopontin expression in a group of lymph node negative breast cancer patients. *Int J Cancer* 79:502-508.
- Tuck AB, Arsenault DM, O'Malley FP, Hota C, Ling MC, Wilson SM, Chambers AF. 1999. Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. *Oncogene* 18:4237-4246.
- van der Pluijm G, Vloedgraven H, Papapoulos S, Löwik C, Grzesik W, Kerr J, Robey PG. 1997. Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. *Lab Invest* 77:665-675.
- van der Voort R, Taher TEI, Keehnen RMJ, Smit L, Groenink M, Pals ST. 1997. Paracrine regulation of germinal center B cell adhesion through the c-Met-hepatocyte growth factor/scatter factor pathway. *J Exp Med* 185:2121-2131.

- Vande Woude GF, Jeffers M, Cortner J, Alvord G, Tsarfaty I, Reseau J. 1997. Met-HGF/SF: Tumorigenesis, invasion and metastasis. *Ciba Found Symp* 212:148-154.
- Wang R, Kobayashi R, Bishop JM. 1996. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. *Proc Natl Acad Sci* 93:8425-8430.
- Wary KK, Mainiero F, Isakoff SJ, Marcantonio EE, Giancotti FG. 1996. The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell* 87:733-743.
- Weber GF, Ashkar S, Glimcher MJ, Cantor H. 1996. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271:509-512.
- Weimar IS, de Jong D, Muller EJ, Nakamura T, Gorp JMH, de Gast GC, Gerritsen WR. 1997. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via  $\alpha 4 \beta 1$  and  $\alpha 5 \beta 1$  integrins. *Blood* 89:990-1000.
- Weimar IS, Miranda N, Muller EJ, Hekman A, Kerst JM, de Gast GC, Gerritsen WR. 1998. Hepatocyte growth factor/scatter factor (HGF/SF) is produced by human bone marrow stromal cells and promotes proliferation, adhesion and survival of human hematopoietic progenitor cells (CD34+). *Exp Hematol* 26:885-894.
- Wong NC, Mueller BM, Barbas CF, Ruminski P, Quaranta V, Lin ECK, Smith JW. 1998.  $\alpha v$  integrins mediate adhesion and migration of breast carcinoma cell lines. *Clin Exp Metastasis* 16:50-61.
- Xuan JW, Hota C, Chambers AF. 1994. Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. *J Cell Biochem* 54:247-255.
- Xuan JW, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ, Chambers AF. 1995. Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. *J Cell Biochem* 57:680-690.
- Yamashita J, Ogawa M, Yamashita S, Nomura K, Kuramoto M, Saishoji T, Shin S. 1994. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res* 54:1630-1633.
- Yao Y, Jin L, Fuchs A, Joseph A, Hastings HM, Goldberg ID, Rosen EM. 1996. Scatter factor protein levels in human breast cancers: Clinicopathological and biological correlations. *Am J Pathol* 149:1707-1717.





Entrez PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Br  
Search PubMed for [Go] [Clear]  
Limits Preview/Index History Clipboard Details

About Entrez

Display Abstract Show: 20 Sort Send to Text

Text Version

☐ 1: J Orthop Res. 1996 Jul;14(4):573-81.

Related Articles, Link

Entrez PubMed

Overview  
Help | FAQ  
Tutorial  
New/Noteworthy  
E-Utilities

PubMed Services

Journals Database  
MeSH Database  
Single Citation Matcher  
Batch Citation Matcher  
Clinical Queries  
LinkOut  
Cubby

Related Resources

Order Documents  
NLM Gateway  
TOXNET  
Consumer Health  
Clinical Alerts  
ClinicalTrials.gov  
PubMed Central

## Spatial distribution of CD44 and hyaluronan in the proximal tibia of the growing rat.

Noonan KJ, Stevens JW, Tammi R, Tammi M, Hernandez JA, Midura RJ.

Department of Orthopaedic Surgery, University of Iowa, College of Medicine, Iowa City 52242, USA.

CD44 has been described as a cell surface hyaluronan receptor present on a variety of different cells, and it is generally assumed to be prevalent in most connective tissues that contain hyaluronan. A major aim of this study was to test that presumption by localizing CD44 and hyaluronan within several tissues of the proximal tibia of the growing rat. Comparison of these profiles would reveal whether CD44 and hyaluronan co-localize with high fidelity, as would be expected if CD44 were a major hyaluronan binding protein. Using in situ hybridization and immunohistochemistry, CD44 was identified on osteoclasts, chondroclasts, osteocytes, hematopoietic marrow cells, synovial cells, and connective tissue fibroblasts (ligaments, tendons, and fascia). Although the majority of osteocytes expressed CD44, reduced expression was observed for osteoblasts and osteoprogenitor cells. Additionally, CD44 was not detected on chondrocytes from epiphyseal and metaphyseal growth cartilages or in meniscal fibrocartilage. Using biotinylated G1 domain from aggrecan and link protein, hyaluronan was observed in the maturational and hypertrophic zones of all growth cartilages, the synovium and other fibroblastic connective tissues, regional areas of the periosteum and endosteum (around osteoblasts, osteoprogenitor cells, and osteoclasts), osteocyte lacunae, and surrounding blood vessels. In regions of co-localization for CD44 and hyaluronan, it seems that CD44 is a likely hyaluronan binding protein in several tissues of the proximal tibia. However, it does not appear to be the predominant hyaluronan binding protein in growing cartilages of the weanling rat.

PMID: 8764866 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

*A Merriam-Webster®*



**Webster's  
Third New  
International  
Dictionary**

UNABRIDGED

**The definitive Merriam-Webster  
unabridged dictionary**





## MOLECULES IN FOCUS

The  $\alpha v\beta 3$  Integrin “Vitronectin Receptor”

MICHAEL A. HORTON

*Department of Medicine, Jules Thorn Institute, The Middlesex Hospital, London W1N 8AA, U.K.*

The  $\alpha v\beta 3$  “vitronectin receptor” is a member of the integrin superfamily of adhesion molecules. As such, this 160/85 kDa heterodimeric protein exhibits many of the typical structural and functional features of integrins. It mediates cell adhesion to extracellular matrix by recognizing the conserved arg-gly-asp (RGD) sequence of several plasma and matrix proteins. Recently, it has also been shown that  $\alpha v\beta 3$  is involved in signal transduction and cell to cell interactions.  $\alpha v\beta 3$  is highly expressed in bone resorbing cells, osteoclasts, and upregulated in response to vascular damage, during angiogenesis and in certain types of malignancy. Antagonists of  $\alpha v\beta 3$  are being developed for use in a variety of diseases associated with altered receptor function or level.

© 1997 Elsevier Science Ltd

Keywords: Integrin Extracellular matrix  $\alpha v\beta 3$  Osteoclast Angiogenesis*Int. J. Biochem. Cell Biol.* (1997) 29, 721–725

## INTRODUCTION

The  $\alpha v\beta 3$  is a member of the integrin superfamily of adhesion proteins. This class of receptors was named in 1986 by Hynes to emphasize their role in integrating the intracellular cytoskeleton with the external milieu. The term “vitronectin receptor” was first applied to  $\alpha v\beta 3$  as it bound the plasma protein vitronectin; it was first purified from placenta and defined as a vitronectin receptor by Pytela in 1985, and cloned and sequenced by Suzuki in 1986. Its name is a misnomer, as it clearly is not selective for vitronectin or its sole receptor.

Vitronectin (S-protein) is a multidomain plasma protein synthesized in liver and involved in a wide range of functions. These include interference with complement activation and blood coagulation; it also integrates with matrix where it is extensively modified and binds proteoglycans. In addition, several integrins are able to recognize vitronectin— $\alpha v\beta 3$ , platelet gpIIb/IIIa ( $\alpha IIb\beta 3$ ) “fibrinogen receptor”, and other  $\alpha v$  integrins,  $\alpha v\beta 5$  and  $\alpha v\beta 1$ .

STRUCTURE OF THE  $\alpha v\beta 3$  INTEGRIN

Integrins (Fig. 1) are heterodimeric membrane glycoproteins. Multiple combinations of the 16  $\alpha$  and eight  $\beta$  subunits occur—these form 21 dimers which define the ligand binding specificity and function of the distinct receptors.

All  $\alpha$  chains show high sequence homology and share common structural features. They vary in size from approximately 120 to 180 kDa and contain seven 60 amino acid long tandem repeats; the C-terminal 4 bind divalent cations via an E-F hand-like structure. Many  $\alpha$  chains, including  $\alpha v$ , are cleaved during biosynthesis, at a conserved site near the trans-membrane domain.  $\beta$  subunits are also homologous to each other, vary from 90 to 110 kDa and have a high cysteine content concentrated mainly in four repeat domains; the N-terminal domain is disulphide bonded to the N-terminus of the  $\beta$  chain.

Structural analysis shows an extended structure with an N-terminal globular head, created by the association of the  $\alpha$  and  $\beta$  chains. Cross-linking studies have confirmed that the  $\alpha$  and  $\beta$  chains are both involved in ligand recognition. The intracellular C-termini of both the  $\alpha$  and  $\beta$  chains interact with the cytoskeleton

and components of the cellular signalling system, such as the kinases FAK, ILK etc. The  $\beta$  chain interaction is well characterized: linkage to the F-actin cytoskeleton, via binding to  $\alpha$ -actinin and talin, has been demonstrated for  $\beta 1$  *in vitro* and this has been confirmed in transfection studies where the cytoplasmic tail domains of  $\beta 3$  and  $\beta 5$  have been exchanged, the  $\beta 3$  tail targeting integrins to focal adhesions.

$\alpha v\beta 3$  purified from tissues typically has a size of approximately 160 ( $\alpha$  chain)/85 ( $\beta$  chain) kDa. On reduction the proteolytic cleavage of the  $\alpha$  chain is revealed by release of a 25 kDa C-terminal fragment and the  $\beta$  chain mobility alters in line with its high cysteine content. cDNA cloning has revealed an  $\alpha v$  chain of 1018 amino acids with 13 potential N-linked glycosylation sites and four typical N-terminal repeat motifs. The  $\alpha v$  gene is located at 2q31-32. The  $\beta 3$  chain is smaller at 762 amino acids, is less heavily glycosylated, and contains 56 cysteine residues, largely in four tandemly repeated domains. The  $\beta 3$  gene is found at 17q21. Several alternatively spliced variants at the C-terminus of the  $\beta 3$  chain have been described, including a unique, secreted, 60 kDa truncated form of unknown function.

#### BIOSYNTHESIS AND TISSUE DISTRIBUTION

The site of highest expression of  $\alpha v\beta 3$  *in vivo* is the osteoclast. Lower levels are also seen in platelets and megakaryocytes, kidney, some vascular smooth muscle, endothelium, and placenta.  $\alpha v\beta 3$  is up-regulated in certain pathologies, such as malignant melanoma, and in numerous *in vitro* cultured adherent cell lines. Non- $\beta 3$ ,  $\alpha v$  integrins are much more widely expressed in normal tissues.

The biosynthesis of  $\alpha v\beta 3$  has not been examined in great detail but studies in melanoma and endothelial cell lines show that it is similar to leucocyte and platelet integrins (LFA and gpIIbIIIa, respectively).  $\alpha$  and  $\beta$  chain synthesis is separately regulated and pulse chase analysis shows that there is an extensive intracellular processing of N-linked carbohydrates, proteolytic cleavage of the  $\alpha v$  chain and  $\alpha$ - $\beta$  chain assembly prior to surface co-expression of the two chains in a biologically active, ligand binding form. There is evidence from cultured cell lines and osteoclast precursors for cytokine regulation of  $\alpha v\beta 3$  expression, as for other integrins.

#### BIOLOGICAL FUNCTIONS

##### Cell-matrix interactions

$\alpha v\beta 3$  mediates the adhesion of cells to vitronectin (vitronectin was known first as "serum spreading factor" and termed vitronectin after its ability to bind glass) and, in a promiscuous manner, to a large number of other extracellular matrix proteins, including fibronectin, fibrinogen and the bone sialoproteins. The key recognition motif is the amino acid triplet arg-gly-aspartate (RGD), which was first demonstrated for the interaction between fibronectin and the  $\alpha 5\beta 1$  integrin by Pierschbacher and Ruoslahti (1984). For certain RGD-containing proteins, for example collagen and laminin, conformational change is required before they are recognized by  $\alpha v\beta 3$ ; this may have functional relevance in the repair and remodelling response in inflammation or tissue injury. In addition,  $\alpha v\beta 3$  has been shown to promote cell migration and provide key signals in the regulation of the balance between cell proliferation and differentiation.

##### Cell-cell interactions

Very recently,  $\alpha v\beta 3$  has been shown to interact with two cell-associated glycoproteins of the immunoglobulin superfamily, CD31 (PCAM-1) and L1/NILE, the latter via an RGD sequence in one of its immunoglobulin domains. Mediation of heterotypic cell-cell adhesion, as for VCAM-1 and  $\alpha 4\beta 1$  and ICAM-1 and  $\alpha m\beta 2$ , suggests that the receptor may be involved in a wider range of physiological and pathological processes than first suspected.

##### Bone resorption

$\alpha v\beta 3$  is the dominant integrin of osteoclasts, bone resorbing cells, both quantitatively and functionally.  $\alpha v\beta 3$  mediates a promiscuous recognition of many RGD-containing bone matrix proteins. Interference with  $\alpha v\beta 3$  using a variety of approaches leads to inhibition of bone resorption.

##### Signal transduction

Aside from the phosphorylation of focal adhesion kinase (FAK) and *src* in focal adhesions, there is increasing evidence for cell signalling operating via integrins, including  $\alpha v\beta 3$ . Thus, endothelial, neutrophil and osteoclast intracellular calcium concentrations

rise as a consequence of contact with RGD-containing proteins and peptides and there is an interrelationship between intracellular calcium levels, cell adhesion, and motility mediated via  $\alpha_v\beta_3$ .

### Angiogenesis

There is increasing evidence that  $\alpha_v$  integrins are upregulated in new capillaries proliferating in response to an angiogenic stimulus. Thus, in

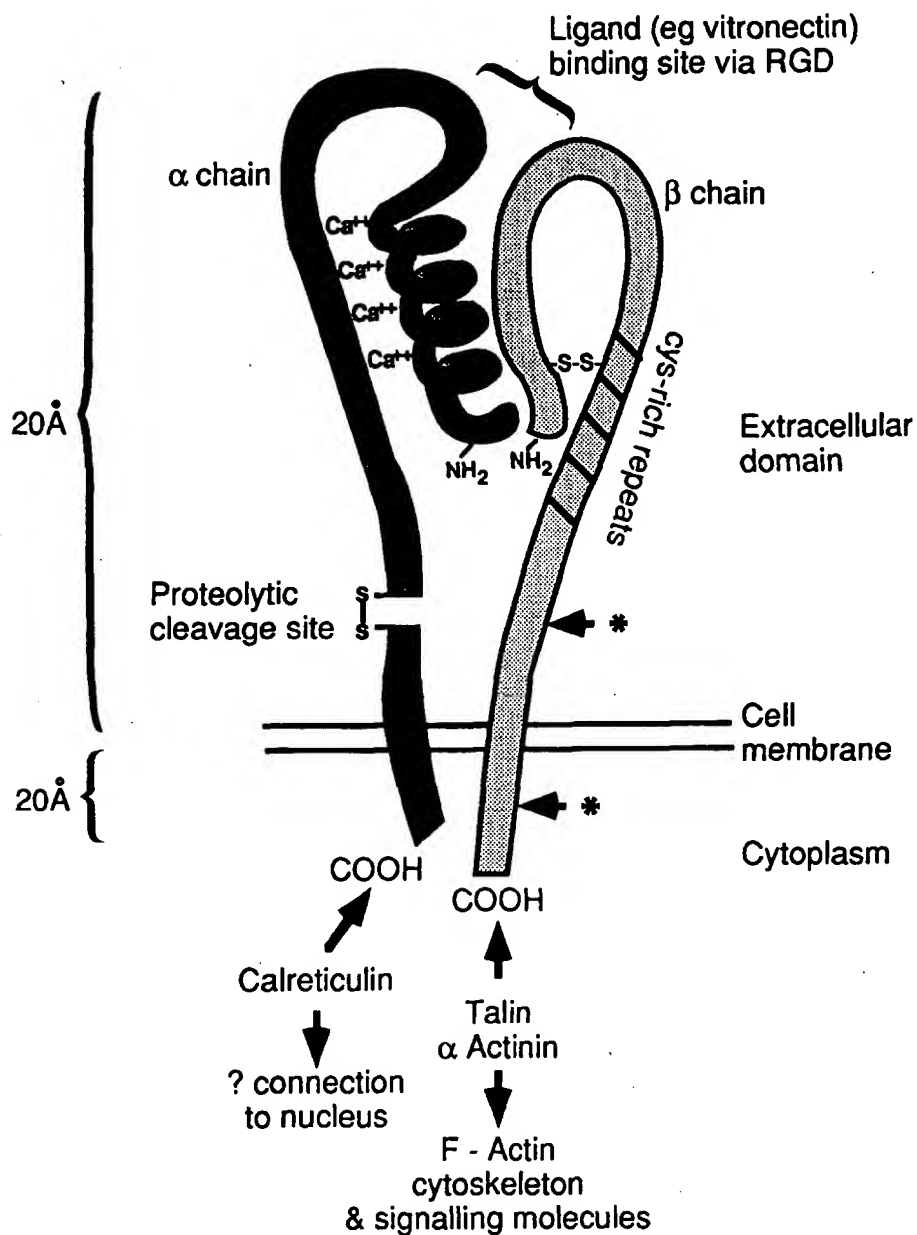


Fig. 1. A stylized view of the  $\alpha_v\beta_3$  "vitronectin receptor" as a model of other integrins.  $\alpha_v\beta_3$  is an  $\alpha\beta$  integrin heterodimer. The  $\alpha$  chain has: four putative divalent cation binding sites near its N-terminus; a proteolytic cleavage site formed during translational modification; a C-terminus that may interact with calreticulin or other cytoskeletal components. The  $\beta$  chain shows a high cysteine content in four tandemly repeated domains; N-terminal disulphide bonding to form a large extracellular loop; a C-terminal interaction with talin and  $\alpha$ -actinin to form a junction with the F-actin cytoskeleton and other signalling molecules; and alternate mRNA splicing (\*) to make either truncated or C-terminal variants of the  $\beta$  chain. The  $\alpha\beta$  heterodimer is a trans-membrane protein with a large N-terminal extracellular domain, the two chains forming a ligand binding site by interaction one with the other. The extracellular domain is of the order of 120 Å units long as evidenced from rotary shadow electron microscopy (see references for further details).

certain pathologies—wound repair, some eye diseases, in association with cancer—the modified phenotype of vascular cells may result in, or be due to, alteration in integrin expression and hence cellular behaviour. There is some controversy as to whether there is a selective activation of  $\alpha v\beta 3$  vs  $\alpha v\beta 5$  (raising the possibility for regulation by distinct growth factors, FGF for  $\beta 3$  and VEGF for  $\beta 5$ ). There is a possibility that  $\alpha v$  integrins may thus be a useful target for diseases characterized by neovascularization.

### Apoptosis

There is evidence for a role for  $\alpha v\beta 3$  in the phagocytosis and removal of some cell types undergoing apoptosis. The best characterized involves the macrophage recognition of senescent neutrophils, an event likely to be important in the limitation of inflammatory tissue injury.

### Development

Recent studies in the mouse have shown that during embryonic development  $\alpha v$  integrins are regulated in two tissues—the central nervous system and skeletal muscle—with expression down-regulated in adult tissues.

### MUTATIONS IN $\alpha v\beta 3$ : IMPLICATIONS FOR RECEPTOR FUNCTION

Mutations in the  $\beta 3$  chain of platelet  $\alpha IIb\beta 3$  (gpIIbIIIa) occur in humans and are known as Glanzmann's syndrome; the condition is characterized by defective platelet aggregation and haemorrhage. There appear to be no major changes in the function of the other integrin receptor utilizing  $\beta 3$ ,  $\alpha v\beta 3$ , either during development or in adult life. It is presumed that this is due to receptor redundancy;  $\alpha v\beta 5$  may complement the deficiency of  $\alpha v\beta 3$  function in, for example, bone. Thus, skeletal abnormality is not a predominant feature of the null  $\beta 3$  gene mutation seen in the Iraqi Jewish form of Glanzmann's syndrome.

An  $\alpha v$  mutation has not been defined in humans. However, an  $\alpha v$  knock-out mouse has recently been described (Hynes, unpublished). Animals survive to birth and, as with Glanzmann's syndrome, skeletal development is normal. Again, the phenotype implies receptor redundancy, but the reasons for tissue selective differences in compensation are unclear.

### MEDICAL AND PHARMACEUTICAL APPLICATIONS

There are three main clinical areas where upregulation of  $\alpha v\beta 3$  has been found in disease and they are being developed as targets for drug development. These are: in angiogenesis in tumours and certain eye diseases such as macular degeneration; in melanoma when it progresses from the horizontal to vertically invasive and metastatic stages; and in coronary arteries following angioplasty leading to vascular restenosis (a humanized  $\beta 3$  monoclonal antibody was protective in the EPIC trial).

To this should be added that bone diseases, such as osteoporosis and bone metastasis, are associated with extensive bone resorption by osteoclasts, cells which are particularly well endowed with  $\alpha v\beta 3$  in their normal state (*vide supra*). Here, *in vivo* data clearly show that osteoclastic resorption can be inhibited by blocking  $\alpha v\beta 3$  function.

The general approach for modifying integrin adhesion receptor behaviour has been to use blocking monoclonal antibodies or agents developed to mimic amino acid motifs in ligands and counter-receptors. It is awaited to see if molecules with specificity for  $\alpha v\beta 3$  are clinically useful. These could be used to inhibit bone resorption in osteoporosis, block coronary artery restenosis, inhibit neovascularization in eye diseases or induced tumour death by depleting its blood supply or by inducing apoptosis, or be used to target melanoma tumour or inhibit metastasis.

*Acknowledgement*—The Wellcome Trust is thanked for supporting this work.

### REFERENCES

- Barclay A. N., Birkeland M. L., Brown M. H., Beyers A. D., Davis S. J., Somoza C. and Williams A. F. (1993) *The Leucocyte Antigen Factsbook*. Academic Press, London.
- Clark E. A. and Brugge J. S. (1995) Integrins and signal transduction pathways: the road taken. *Science* **268**, 233–239.
- Felding-Habermann B. and Cheresch D. A. (1993) Vitronectin and its receptors. *Curr. Opin. Cell Biol.* **5**, 864–868.
- Friedlander M., Brooks P. C., Shaffer R. W., Kincaid C. M., Varner J. A. and Cheresch D. A. (1995) Definition of two angiogenic pathways by distinct integrins. *Science* **270**, 1500–1502.
- Gadek T. and Blackburn B. K. (1995) Identification and development of integrin/ligand antagonists for the treatment of human disease. In *Adhesion Receptors as Therapeutic Targets* (Edited by Horton M. A.), pp. 247–272. CRC Press, Boca Raton.

- Horton M. A. (1990) Vitronectin receptor: tissue specific expression or adaptation to culture? *Int. J. Exp. Pathol.* **71**, 741-759.
- Horton M. A. and Rodan G. A. (1995) Integrins as therapeutic targets in bone disease. In *Adhesion Receptors as Therapeutic Targets* (Edited by Horton M. A.), pp. 223-245. CRC Press, Boca Raton.
- Hynes R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.
- Nesbit M. and Herlyn M. (1994) Adhesion receptors in human melanoma progression. *Invas. Metast.* **14**, 131-146.
- Pierschbacher M. D. and Ruoslahti E. (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33.
- Pytela R., Pierschbacher M. D. and Ruoslahti E. (1985) *Proc. Natl Acad. Sci., U.S.A.* **82**, 5766-5770.
- Suzuki S., Argraves W. S., Pytela R., Arai H., Krusius T., Pierschbacher, M. D. and Ruoslahti E. (1986) *Proc. Natl Acad. Sci., U.S.A.* **83**, 8614-8618.
- Tamkun J. W., DeSimone D. W., Fonda D., Patel R. S., Buck C., Horwitz A. F. and Hynes R. O. (1986) *Cell* **46**, 271-282.
- Topol E. J. (1994) Randomised trial of coronary intervention with antibody against platelet IIb/IIIa integrin for reduction of clinical restenosis: results at six months. *Lancet* **343**, 881-886.





# Blast 2 Sequences results

PubMed

Entrez

BLAST

OMIM

Taxonomy

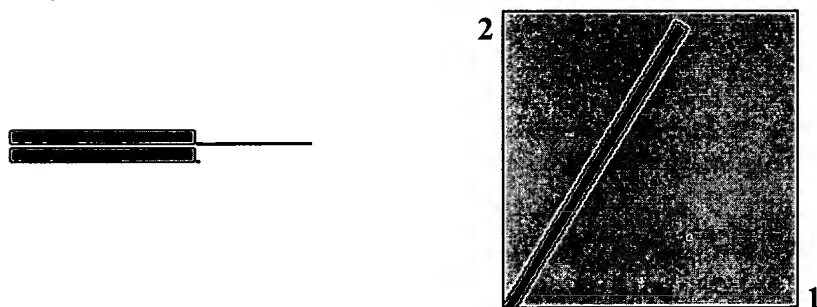
Structure

## BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.10 [Oct-19-2004]

Matrix **BLOSUM62** gap open: **11** gap extension: **1**  
 x\_dropoff: **50** expect: **10.000** wordsize: **3** Filter ☒ **Align**

Sequence 1 lc|seq\_1 Length 63 (1 .. 63) SEQ ID NO: 11

Sequence 2 lc|seq\_2 Length 40 (1 .. 40) SEQ ID NO: 15



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 79.0 bits (193), Expect = 4e-14  
 Identities = 38/39 (97%), Positives = 38/39 (97%)

Query: 1 RSRRATEVFTPVVPTVDITYDGRGDSVVYGRRSKSKKFRR 39  
 RSRRATEVFTPVVPTVDITYDGRGDSVVYGRSKSKKFRR  
 Sbjct: 1 RSRRATEVFTPVVPTVDITYDGRGDSVVYGLRSKSKKFRR 39

CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda K H  
 0.320 0.135 0.386

Gapped  
 Lambda K H  
 0.267 0.0410 0.140

Matrix: BLOSUM62

Gap Penalties: Existence: 11, Extension: 1

Number of Sequences: 1

Number of Hits to DB: 51

Number of extensions: 11

Number of successful extensions: 1

Number of sequences better than 10.0: 1

Number of HSP's better than 10.0 without gapping: 1

Number of HSP's gapped: 1

Number of HSP's successfully gapped: 1

Number of extra gapped extensions for HSPs above 10.0: 0

Length of query: 63

Length of database: 837,004,805  
Length adjustment: 38  
Effective length of query: 25  
Effective length of database: 837,004,767  
Effective search space: 20925119175  
Effective search space used: 20925119175  
Neighboring words threshold: 9  
Window for multiple hits: 0  
X1: 16 ( 7.4 bits)  
X2: 129 (49.7 bits)  
X3: 129 (49.7 bits)  
S1: 41 (21.8 bits)  
S2: 69 (31.2 bits)



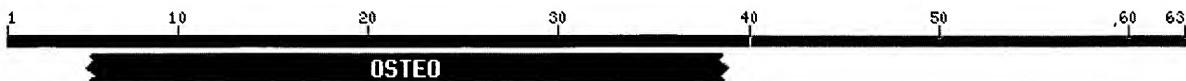
## NCBI Conserved Domain Search

[New Search](#)[PubMed](#)[Nucleotide](#)[Protein](#)[Structure](#)[CDD](#)[Taxonomy](#)[Help?](#)

RPS-BLAST 2.2.10 [Oct-19-2004]

Query= local sequence:  
(63 letters) **SEQ70NO:11**

Database: cdd.v2.03

[Click on boxes for multiple alignments](#)

Domain Relatives

PSSMs producing significant alignments:

Score E  
(bits) value[gnl|CDD|11 smart00017](#), OSTEO, Osteopontin; Osteopontin is an acidic phosph... [57.1](#) 5e-10

[gnl|CDD|11 smart00017](#), OSTEO, Osteopontin; Osteopontin is an acidic phosphorylated glycoprotein of about 40 Kd which is abundant in the mineral matrix of bones and which binds tightly to hydroxyapatite. It is suggested that osteopontin might function as a cell attachment factor and could play a key role in the adhesion of osteoclasts to the mineral matrix of bone .

CD-Length = 286 residues, only 12.2% aligned  
Score = 57.1 bits (137), Expect = 5e-10Query: 5 ATEVFTPVVPTVDTYDGRGDSVVYGRRSKSKKFRR 39  
Sbjct: 119 AATVFTPFPVPTVDTNDGRGDSVAYGLRSKSKVFKV 153

**Citing CD-Search:** Marchler-Bauer A, Bryant SH (2004), "*CD-Search: protein domain annotations on the fly*.", *Nucleic Acids Res.* **32**:W327-331.

[Help](#) | [Disclaimer](#) | [Write to the Help Desk](#)  
[NCBI](#) | [NLM](#) | [NIH](#)



# results of BLAST

BLASTP 2.2.11 [May-08-2005]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1116880158-24786-155013903519.BLASTQ3

Query=

(63 letters) SEQ ID NO: 11

Database: All non-redundant GenBank CDS

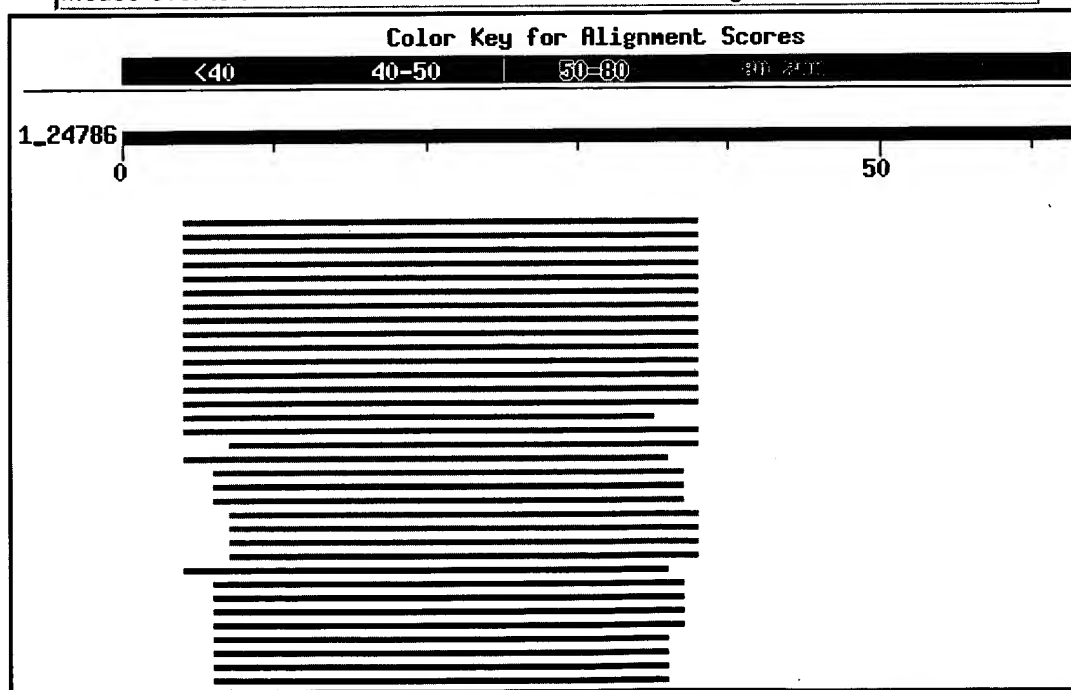
translations+PDB+SwissProt+PIR+PRF excluding environmental samples  
2,470,458 sequences; 837,004,805 total letters

If you have any problems or questions with the results of this search please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)

## Distribution of 34 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments



Sequences producing significant alignments:			Score (bits)	E Value	
<a href="#">gi 992950 dbj BAA05951.1 </a>	OPN-c [Homo sapiens]		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 992949 dbj BAA05950.1 </a>	OPN-b [Homo sapiens]		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 992948 dbj BAA05949.1 </a>	OPN-a [Homo sapiens]		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 60817593 gb AAX36430.1 </a>	secreted phosphoprotein 1 [synth...		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 16924233 gb AAH17387.1 </a>	SPP1 protein [Homo sapiens] >gi ...		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 62205271 gb AAH93033.1 </a>	SPP1 protein [Homo sapiens]		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 30583805 gb AAP36151.1 </a>	Homo sapiens secreted phosphopro...		<u>72</u>	6e-12	
<a href="#">gi 55622886 ref XP_517332.1 </a>	PREDICTED: similar to Osteopon...		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 54697144 gb AAV38944.1 </a>	secreted phosphoprotein 1 (osteo...		<u>72</u>	6e-12	
<a href="#">gi 54697142 gb AAV38943.1 </a>	secreted phosphoprotein 1 (osteo...		<u>72</u>	6e-12	
<a href="#">gi 55730563 emb CAH92003.1 </a>	hypothetical protein [Pongo pyg...		<u>72</u>	6e-12	
<a href="#">gi 22761565 dbj BAC11635.1 </a>	unnamed protein product [Homo s...		<u>72</u>	6e-12	<b>G</b>
<a href="#">gi 47522868 ref NP_999188.1 </a>	secreted phosphoprotein-I [Sus...		<u>54</u>	2e-06	<b>G</b>
<a href="#">gi 6448616 emb CAB61259.1 </a>	osteopontin [Sus scrofa]		<u>54</u>	2e-06	
<a href="#">gi 57109122 ref XP_535649.1 </a>	PREDICTED: similar to Osteopon...		<u>53</u>	2e-06	<b>G</b>
<a href="#">gi 58568401 gb AAW78987.1 </a>	GekBS141P [Gekko japonicus]		<u>52</u>	5e-06	
<a href="#">gi 57163973 ref NP_001009224.1 </a>	osteopontin [Ovis aries] >g...		<u>51</u>	8e-06	<b>G</b>
<a href="#">gi 280933 pir  JC1191</a>	osteopontin precursor - rabbit >gi 21...		<u>51</u>	1e-05	
<a href="#">gi 27806401 ref NP_776612.1 </a>	secreted phosphoprotein 1 [Bos...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 12805293 gb AAH02113.1 </a>	Spp1 protein [Mus musculus] >gi ...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 15679955 gb AAH14284.1 </a>	Spp1 protein [Mus musculus]		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 19774215 gb AAL99081.1 </a>	osteopontin [Bos taurus] >gi 620...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 61553509 gb AAX46418.1 </a>	secreted phosphoprotein 1 (osteo...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 89697 pir  JS0638</a>	osteopontin precursor - bovine		<u>50</u>	2e-05	
<a href="#">gi 9800644 gb AAB34351.2 </a>	early T-lymphocyte activator-1; o...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 538245 dbj BAA03980.1 </a>	secreted phosphoprotein-1 precurs...		<u>49</u>	5e-05	
<a href="#">gi 200158 gb AAA57265.1 </a>	osteopontin precursor [Mus musculus]		<u>48</u>	7e-05	<b>G</b>
<a href="#">gi 297546 emb CAA36132.1 </a>	osteopontin [Mus musculus]		<u>48</u>	7e-05	<b>G</b>
<a href="#">gi 6678113 ref NP_033289.1 </a>	secreted phosphoprotein 1 [Mus ...		<u>48</u>	7e-05	<b>G</b>
<a href="#">gi 34786065 gb AAH57858.1 </a>	Spp1 protein [Mus musculus] >gi ...		<u>48</u>	7e-05	<b>G</b>
<a href="#">gi 50925511 gb AAH78874.1 </a>	Secreted phosphoprotein 1 [Rattu...		<u>47</u>	1e-04	<b>G</b>
<a href="#">gi 205860 gb AAA41762.1 </a>	osteopontin precursor >gi 6981580 ...		<u>47</u>	1e-04	<b>G</b>
<a href="#">gi 7441835 pir  JC5811</a>	osteopontin - rat		<u>47</u>	1e-04	
<a href="#">gi 1871124 dbj BAA19247.1 </a>	osteopontin [Rattus norvegicus]		<u>47</u>	1e-04	<b>G</b>

## Alignments

Get selected sequences

Select all

Deselect all

☐ >gi|992950|dbj|BAA05951.1| **G** OPN-c [Homo sapiens]  
Length = 287

Score = 71.6 bits (174), Expect = 6e-12  
Identities = 34/35 (97%), Positives = 34/35 (97%)

Query: 5 ATEVFTPVVPTVDTYDGRGDSVVYGRRSKSKKFRR 39



# NCBI Conserved Domain Search

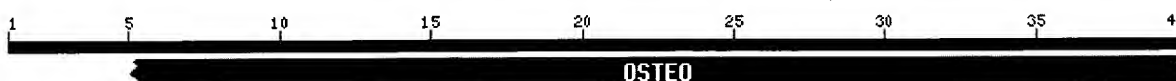
[New Search](#)
[PubMed](#)
[Nucleotide](#)
[Protein](#)
[Structure](#)
[CDD](#)
[Taxonomy](#)
[Help?](#)

RPS-BLAST 2.2.10 [Oct-19-2004]

Query= local sequence:

(40 letters) **SEQ ID NO: 15**

Database: cdd.v2.03


 Domain Relatives

PSSMs producing significant alignments:

 Score E  
(bits) value

[gnl|CDD|11 smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosph...](#) **60.6** 4e-11

[gnl|CDD|11 smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosphorylated glycoprotein of about 40 Kd which is abundant in the mineral matrix of bones and which binds tightly to hydroxyapatite. It is suggested that osteopontin might function as a cell attachment factor and could play a key role in the adhesion of osteoclasts to the mineral matrix of bone .](#)

CD-Length = 286 residues, only 12.6% aligned  
Score = 60.6 bits (146), Expect = 4e-11

Query: 5 ATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRP 40  
Sbjct: 119 AATVFTPFPVPTVDTNDGRGDSVAYGLRSKSKVFKVS 154

**Citing CD-Search:** Marchler-Bauer A, Bryant SH (2004), "*CD-Search: protein domain annotations on the fly.*", *Nucleic Acids Res.* **32**:W327-331.

[Help](#) | [Disclaimer](#) | [Write to the Help Desk](#)  
[NCBI](#) | [NLM](#) | [NIH](#)



# results of BLAST

BLASTP 2.2.11 [May-08-2005]

**Reference:**

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1116880484-2802-168172056308.BLASTQ3

**Query=**

(40 letters) SEQFON015

**Database:** All non-redundant GenBank CDS

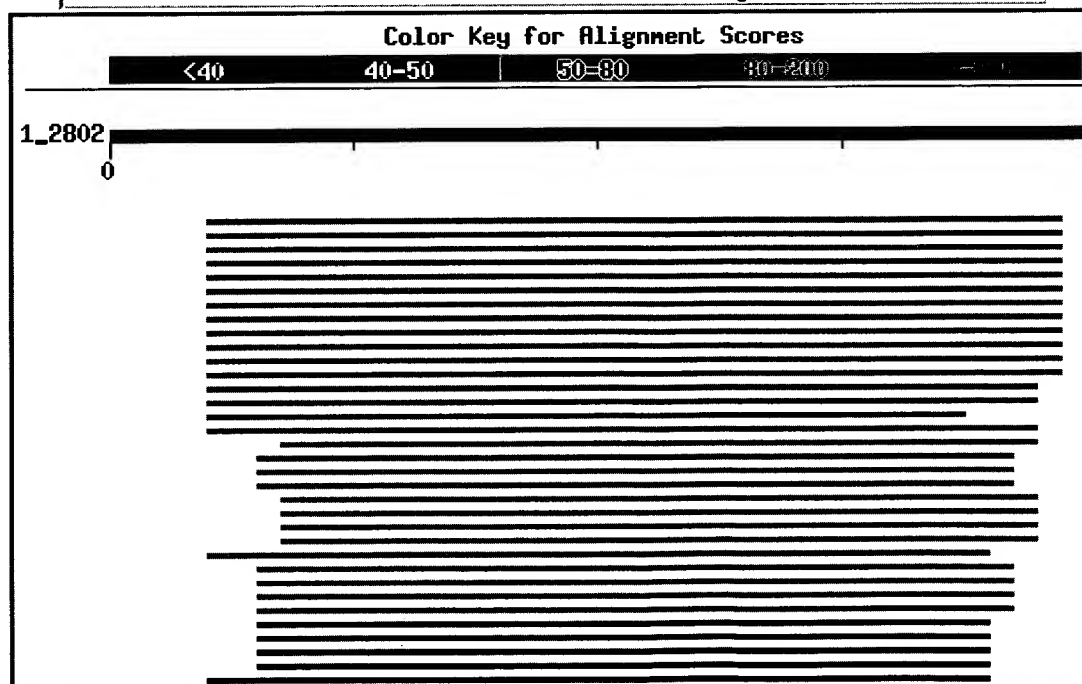
translations+PDB+SwissProt+PIR+PRF excluding environmental samples  
2,470,458 sequences; 837,004,805 total letters

If you have any problems or questions with the results of this search  
please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)

## Distribution of 34 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments



Sequences producing significant alignments:			Score (bits)	E Value	
<a href="#">gi 992950 dbj BAA05951.1 </a>	OPN-c [Homo sapiens]		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 992949 dbj BAA05950.1 </a>	OPN-b [Homo sapiens]		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 992948 dbj BAA05949.1 </a>	OPN-a [Homo sapiens]		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 60817593 gb AAX36430.1 </a>	secreted phosphoprotein 1 [synth...		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 16924233 gb AAH17387.1 </a>	SPP1 protein [Homo sapiens] >gi ...		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 62205271 gb AAH93033.1 </a>	SPP1 protein [Homo sapiens]		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 30583805 gb AAP36151.1 </a>	Homo sapiens secreted phosphopro...		<u>77</u>	2e-13	
<a href="#">gi 55622886 ref XP_517332.1 </a>	PREDICTED: similar to Osteopon...		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 54697144 gb AAV38944.1 </a>	secreted phosphoprotein 1 (osteo...		<u>77</u>	2e-13	
<a href="#">gi 54697142 gb AAV38943.1 </a>	secreted phosphoprotein 1 (osteo...		<u>77</u>	2e-13	
<a href="#">gi 55730563 emb CAH92003.1 </a>	hypothetical protein [Pongo pyg...		<u>77</u>	2e-13	
<a href="#">gi 22761565 dbj BAC11635.1 </a>	unnamed protein product [Homo s...		<u>77</u>	2e-13	<b>G</b>
<a href="#">gi 47522868 ref NP_999188.1 </a>	secreted phosphoprotein-I [Sus...		<u>56</u>	3e-07	<b>G</b>
<a href="#">gi 6448616 emb CAB61259.1 </a>	osteopontin [Sus scrofa]		<u>56</u>	3e-07	
<a href="#">gi 57109122 ref XP_535649.1 </a>	PREDICTED: similar to Osteopon...		<u>55</u>	4e-07	<b>G</b>
<a href="#">gi 58568401 gb AAW78987.1 </a>	GekBS141P [Gekko japonicus]		<u>54</u>	9e-07	
<a href="#">gi 57163973 ref NP_001009224.1 </a>	osteopontin [Ovis aries] >g...		<u>54</u>	2e-06	<b>G</b>
<a href="#">gi 27806401 ref NP_776612.1 </a>	secreted phosphoprotein 1 [Bos...		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 12805293 gb AAH02113.1 </a>	Spp1 protein [Mus musculus] >gi ...		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 15679955 gb AAH14284.1 </a>	Spp1 protein [Mus musculus]		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 19774215 gb AAL99081.1 </a>	osteopontin [Bos taurus] >gi 620...		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 61553509 gb AAX46418.1 </a>	secreted phosphoprotein 1 (osteo...		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 89697 pir  JS0638</a>	osteopontin precursor - bovine		<u>52</u>	4e-06	
<a href="#">gi 9800644 gb AAB34351.2 </a>	early T-lymphocyte activator-1; o...		<u>52</u>	4e-06	<b>G</b>
<a href="#">gi 280933 pir  JC1191</a>	osteopontin precursor - rabbit >gi 21...		<u>52</u>	6e-06	
<a href="#">gi 200158 gb AAA57265.1 </a>	osteopontin precursor [Mus musculus]		<u>50</u>	1e-05	<b>G</b>
<a href="#">gi 297546 emb CAA36132.1 </a>	osteopontin [Mus musculus]		<u>50</u>	1e-05	<b>G</b>
<a href="#">gi 6678113 ref NP_033289.1 </a>	secreted phosphoprotein 1 [Mus ...		<u>50</u>	1e-05	<b>G</b>
<a href="#">gi 34786065 gb AAH57858.1 </a>	Spp1 protein [Mus musculus] >gi ...		<u>50</u>	1e-05	<b>G</b>
<a href="#">gi 50925511 gb AAH78874.1 </a>	Secreted phosphoprotein 1 [Rattu...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 205860 gb AAA41762.1 </a>	osteopontin precursor >gi 6981580 ...		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 7441835 pir  JC5811</a>	osteopontin - rat		<u>50</u>	2e-05	
<a href="#">gi 1871124 dbj BAA19247.1 </a>	osteopontin [Rattus norvegicus]		<u>50</u>	2e-05	<b>G</b>
<a href="#">gi 538245 dbj BAA03980.1 </a>	secreted phosphoprotein-1 precurs...		<u>49</u>	3e-05	

# Alignments

Get selected sequences

Select all

Deselect all

☐ >[gi|992950|dbj|BAA05951.1|](#) **G** OPN-c [Homo sapiens]  
Length = 287

Score = 76.6 bits (187), Expect = 2e-13  
Identities = 36/36 (100%), Positives = 36/36 (100%)

Query: 5 ATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRP 40





# results of BLAST

BLASTP 2.2.11 [May-08-2005]

**Reference:**

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1116880158-24786-155013903519.BLASTQ3

**Query=**

(63 letters) SEQ ID NO: 11

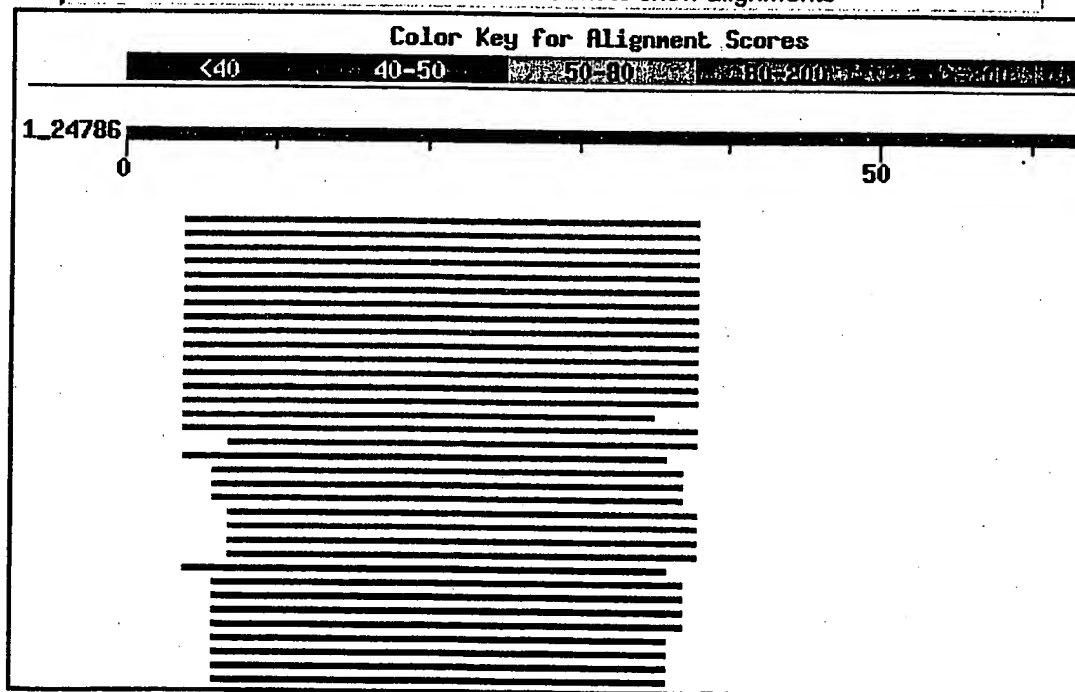
**Database:** All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples  
2,470,458 sequences; 837,004,805 total letters

If you have any problems or questions with the results of this search please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)

## Distribution of 34 Blast Hits on the Query Sequence

Mouse-over to show define and scores. Click to show alignments



## Sequences producing significant alignments:

	Score (bits)	E Value	
<a href="#">gi 992950 dbj BAA05951.1</a> OPN-c [Homo sapiens]	72	6e-12	<b>G</b>
<a href="#">gi 992949 dbj BAA05950.1</a> OPN-b [Homo sapiens]	72	6e-12	<b>G</b>
<a href="#">gi 992948 dbj BAA05949.1</a> OPN-a [Homo sapiens]	72	6e-12	<b>G</b>
<a href="#">gi 60817593 qb AAX36430.1</a> secreted phosphoprotein 1 [synth...	72	6e-12	<b>G</b>
<a href="#">gi 16924233 qb AAH17387.1</a> SPP1 protein [Homo sapiens] >gi ...	72	6e-12	<b>G</b>
<a href="#">gi 62205271 qb AAH93033.1</a> SPP1 protein [Homo sapiens]	72	6e-12	<b>G</b>
<a href="#">gi 30583805 qb AAP36151.1</a> Homo sapiens secreted phosphopro...	72	6e-12	
<a href="#">gi 55622886 ref XP_517332.1</a> PREDICTED: similar to Osteopon...	72	6e-12	<b>G</b>
<a href="#">gi 54697144 qb AAV38944.1</a> secreted phosphoprotein 1 (osteo...	72	6e-12	
<a href="#">gi 54697142 qb AAV38943.1</a> secreted phosphoprotein 1 (osteo...	72	6e-12	
<a href="#">gi 55730563 emb CAH92003.1</a> hypothetical protein [Pongo pyg...	72	6e-12	
<a href="#">gi 22761565 dbj BAC11635.1</a> unnamed protein product [Homo s...	72	6e-12	<b>G</b>
<a href="#">gi 47522868 ref NP_999188.1</a> secreted phosphoprotein-I [Sus...	54	2e-06	<b>G</b>
<a href="#">gi 6448616 emb CAB61259.1</a> osteopontin [Sus scrofa]	54	2e-06	
<a href="#">gi 57109122 ref XP_535649.1</a> PREDICTED: similar to Osteopon...	53	2e-06	<b>G</b>
<a href="#">gi 58568401 qb AAW78987.1</a> GekBS141P [Gekko japonicus]	52	5e-06	
<a href="#">gi 57163973 ref NP_001009224.1</a> osteopontin [Ovis aries] >g...	51	8e-06	<b>G</b>
<a href="#">gi 280933 pir  JC1191</a> osteopontin precursor - rabbit >gi 21...	51	1e-05	
<a href="#">gi 27806401 ref NP_776612.1</a> secreted phosphoprotein 1 [Bos...	50	2e-05	<b>G</b>
<a href="#">gi 12805293 qb AAH02113.1</a> Sppl protein [Mus musculus] >gi ...	50	2e-05	<b>G</b>
<a href="#">gi 15679955 qb AAH14284.1</a> Sppl protein [Mus musculus]	50	2e-05	<b>G</b>
<a href="#">gi 19774215 qb AAL99081.1</a> osteopontin [Bos taurus] >gi 620...	50	2e-05	<b>G</b>
<a href="#">gi 61553509 qb AAX46418.1</a> secreted phosphoprotein 1 (osteo...	50	2e-05	<b>G</b>
<a href="#">gi 89697 pir  JS0638</a> osteopontin precursor - bovine	50	2e-05	
<a href="#">gi 9800644 qb AAB34351.2</a> early T-lymphocyte activator-1; o...	50	2e-05	<b>G</b>
<a href="#">gi 538245 dbj BAA03980.1</a> secreted phosphoprotein-1 precurs...	49	5e-05	
<a href="#">gi 200158 qb AAA57265.1</a> osteopontin precursor [Mus musculus]	48	7e-05	<b>G</b>
<a href="#">gi 297546 emb CAA36132.1</a> osteopontin [Mus musculus]	48	7e-05	<b>G</b>
<a href="#">gi 6678113 ref NP_033289.1</a> secreted phosphoprotein 1 [Mus ...	48	7e-05	<b>G</b>
<a href="#">gi 34786065 qb AAH57858.1</a> Sppl protein [Mus musculus] >gi ...	48	7e-05	<b>G</b>
<a href="#">gi 50925511 qb AAH78874.1</a> Secreted phosphoprotein 1 [Rattu...	47	1e-04	<b>G</b>
<a href="#">gi 205860 qb AAA41762.1</a> osteopontin precursor >gi 6981580 ...	47	1e-04	<b>G</b>
<a href="#">gi 7441835 pir  JC5811</a> osteopontin - rat	47	1e-04	
<a href="#">gi 1871124 dbj BAA19247.1</a> osteopontin [Rattus norvegicus]	47	1e-04	<b>G</b>

## Alignments

Get selected sequences

Select all

Deselect all

☐ >[gi|992950|dbj|BAA05951.1](#) **G** OPN-c [Homo sapiens]  
Length = 287

Score = 71.6 bits (174), Expect = 6e-12  
Identities = 34/35 (97%), Positives = 34/35 (97%)

Query: 5 ATEVFPTPVVPTVDITYDGRGDSVVYGRRSKSKKFRR 39



# results of BLAST

BLASTP 2.2.11 [May-08-2005]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1116880484-2802-168172056308.BLASTQ3

Query=

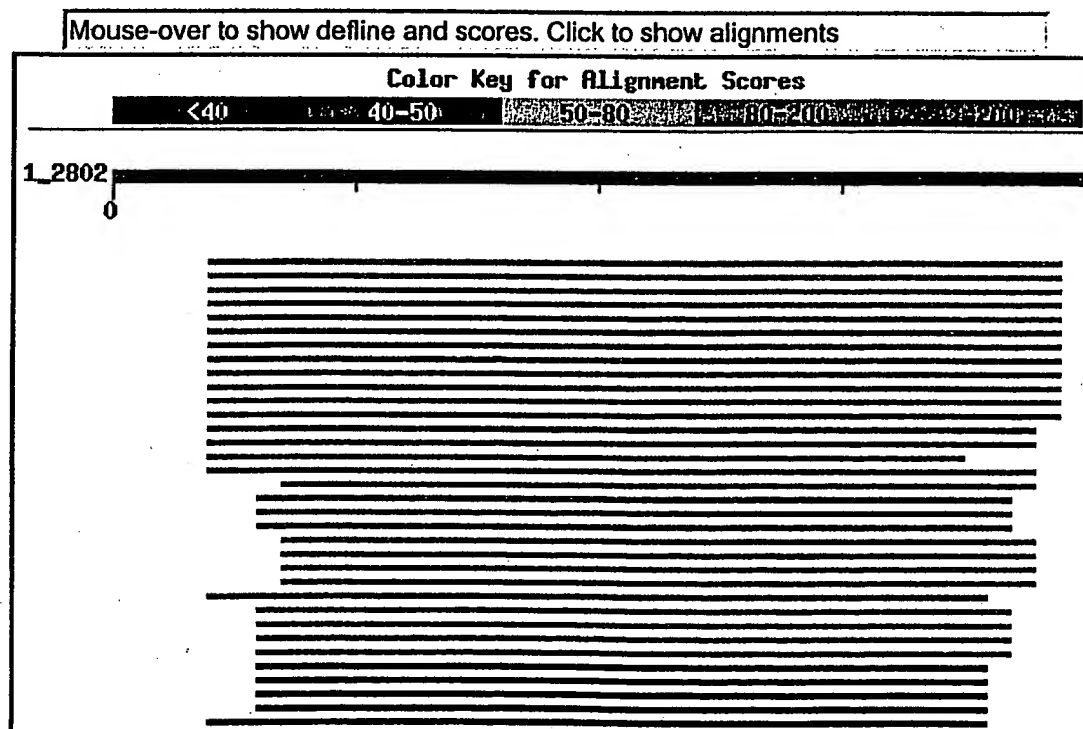
(40 letters) SEATON015

Database: All non-redundant GenBank CDS  
translations+PDB+SwissProt+PIR+PRF excluding environmental samples  
2,470,458 sequences; 837,004,805 total letters

If you have any problems or questions with the results of this search  
please refer to the [BLAST FAQs](#)

[Taxonomy reports](#)

## Distribution of 34 Blast Hits on the Query Sequence



## Sequences producing significant alignments:

		Score (bits)	E Value	
<a href="#">gi 992950 dbj BAA05951.1 </a>	OPN-c [Homo sapiens]	77	2e-13	<b>G</b>
<a href="#">gi 992949 dbj BAA05950.1 </a>	OPN-b [Homo sapiens]	77	2e-13	<b>G</b>
<a href="#">gi 992948 dbj BAA05949.1 </a>	OPN-a [Homo sapiens]	77	2e-13	<b>G</b>
<a href="#">gi 60817593 qb AAX36430.1 </a>	secreted phosphoprotein 1 [synth...	77	2e-13	<b>G</b>
<a href="#">gi 16924233 qb AAH17387.1 </a>	SPP1 protein [Homo sapiens] >gi ...	77	2e-13	<b>G</b>
<a href="#">gi 62205271 qb AAH93033.1 </a>	SPP1 protein [Homo sapiens]	77	2e-13	<b>G</b>
<a href="#">gi 30583805 qb AAP36151.1 </a>	Homo sapiens secreted phosphopro...	77	2e-13	
<a href="#">gi 55622886 ref XP_517332.1 </a>	PREDICTED: similar to Osteopon...	77	2e-13	<b>G</b>
<a href="#">gi 54697144 qb AAV38944.1 </a>	secreted phosphoprotein 1 (osteo...	77	2e-13	
<a href="#">gi 54697142 qb AAV38943.1 </a>	secreted phosphoprotein 1 (osteo...	77	2e-13	
<a href="#">gi 55730563 emb CAH92003.1 </a>	hypothetical protein [Pongo pyg...	77	2e-13	
<a href="#">gi 22761565 dbj BAC11635.1 </a>	unnamed protein product [Homo s...	77	2e-13	<b>G</b>
<a href="#">gi 47522868 ref NP_999188.1 </a>	secreted phosphoprotein-I [Sus...	56	3e-07	<b>G</b>
<a href="#">gi 6448616 emb CAB61259.1 </a>	osteopontin [Sus scrofa]	56	3e-07	
<a href="#">gi 57109122 ref XP_535649.1 </a>	PREDICTED: similar to Osteopon...	55	4e-07	<b>G</b>
<a href="#">gi 58568401 qb AAW78987.1 </a>	GekBS141P [Gekko japonicus]	54	9e-07	
<a href="#">gi 57163973 ref NP_001009224.1 </a>	osteopontin [Ovis aries] >g...	54	2e-06	<b>G</b>
<a href="#">gi 27806401 ref NP_776612.1 </a>	secreted phosphoprotein 1 [Bos...	52	4e-06	<b>G</b>
<a href="#">gi 12805293 qb AAH02113.1 </a>	Sppl protein [Mus musculus] >gi ...	52	4e-06	<b>G</b>
<a href="#">gi 15679955 qb AAH14284.1 </a>	Sppl protein [Mus musculus]	52	4e-06	<b>G</b>
<a href="#">gi 19774215 qb AAL99081.1 </a>	osteopontin [Bos taurus] >gi 620...	52	4e-06	<b>G</b>
<a href="#">gi 61553509 qb AAX46418.1 </a>	secreted phosphoprotein 1 (osteo...	52	4e-06	<b>G</b>
<a href="#">gi 89697 pir  JS0638</a>	osteopontin precursor - bovine	52	4e-06	
<a href="#">gi 9800644 qb AAB34351.2 </a>	early T-lymphocyte activator-1; o...	52	4e-06	<b>G</b>
<a href="#">gi 280933 pir  JC1191</a>	osteopontin precursor - rabbit >gi 21...	52	6e-06	
<a href="#">gi 200158 qb AAA57265.1 </a>	osteopontin precursor [Mus musculus]	50	1e-05	<b>G</b>
<a href="#">gi 297546 emb CAA36132.1 </a>	osteopontin [Mus musculus]	50	1e-05	<b>G</b>
<a href="#">gi 6678113 ref NP_033289.1 </a>	secreted phosphoprotein 1 [Mus ...	50	1e-05	<b>G</b>
<a href="#">gi 34786065 qb AAH57858.1 </a>	Sppl protein [Mus musculus] >gi ...	50	1e-05	<b>G</b>
<a href="#">gi 50925511 qb AAH78874.1 </a>	Secreted phosphoprotein 1 [Rattu...	50	2e-05	<b>G</b>
<a href="#">gi 205860 qb AAA41762.1 </a>	osteopontin precursor >gi 6981580 ...	50	2e-05	<b>G</b>
<a href="#">gi 7441835 pir  JC5811</a>	osteopontin - rat	50	2e-05	
<a href="#">gi 1871124 dbj BAA19247.1 </a>	osteopontin [Rattus norvegicus]	50	2e-05	<b>G</b>
<a href="#">gi 538245 dbj BAA03980.1 </a>	secreted phosphoprotein-1 precurs...	49	3e-05	

## Alignments

Get selected sequences

Select all

Deselect all

☐ >gi|992950|dbj|BAA05951.1| **G** OPN-c [Homo sapiens]  
Length = 287

Score = 76.6 bits (187), Expect = 2e-13  
Identities = 36/36 (100%), Positives = 36/36 (100%)

Query: 5 ATEVFTPVVPTVDITYDGRGDSVVYGLRSKSKKFRRP 40



NCBI

## NCBI Conserved Domain Search

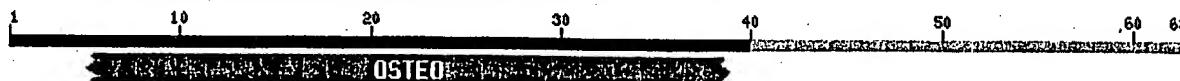
[New Search](#)[PubMed](#)[Nucleotide](#)[Protein](#)[Structure](#)[CDD](#)[Taxonomy](#)[Help?](#)

RPS-BLAST 2.2.10 [Oct-19-2004]

Query= local sequence:

(63 letters) SEQIDNO:11

Database: cdd.v2.03

[Click on boxes for multiple alignments](#)[Show](#) Domain Relatives

PSSMs producing significant alignments:

Score E  
(bits) value[gnl|CDD|11](#) smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosp... 57.1 5e-10

[gnl|CDD|11](#) smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosphorylated glycoprotein of about 40 Kd which is abundant in the mineral matrix of bones and which binds tightly to hydroxyapatite. It is suggested that osteopontin might function as a cell attachment factor and could play a key role in the adhesion of osteoclasts to the mineral matrix of bone .

CD-Length = 286 residues, only 12.2% aligned  
Score = 57.1 bits (137), Expect = 5e-10

Query: 5 ATEVFPTPVVPTVD TYDGRGDSVVYGRRSKSKKFR 39  
Sbjct: 119 AATVFPTPEVPTVD TNDGRGDSVAYGLRSKSKVFKV 153

**Citing CD-Search:** Marchler-Bauer A, Bryant SH (2004), "CD-Search: protein domain annotations on the fly.", *Nucleic Acids Res.* 32:W327-331.

[Help](#) | [Disclaimer](#) | [Write to the Help Desk](#)  
[NCBI](#) | [NLM](#) | [NIH](#)



NCBI

## NCBI Conserved Domain Search

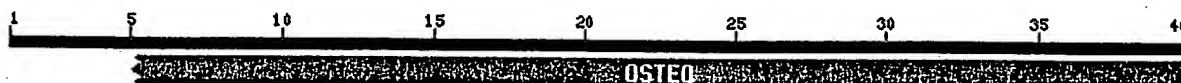
[New Search](#)[PubMed](#)[Nucleotide](#)[Protein](#)[Structure](#)[CDD](#)[Taxonomy](#)[Help?](#)

RPS-BLAST 2.2.10 [Oct-19-2004]

Query= local sequence:

(40 letters) SEQ ID NO: 15

Database: cdd.v2.03

[Click on boxes for multiple alignments](#)[Show](#)

Domain Relatives

PSSMs producing significant alignments:

Score E  
(bits) value[gnl|CDD|11](#) smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosph... 60.6 4e-11

[gnl|CDD|11](#) smart00017, OSTEO, Osteopontin; Osteopontin is an acidic phosphorylated glycoprotein of about 40 Kd which is abundant in the mineral matrix of bones and which binds tightly to hydroxyapatite. It is suggested that osteopontin might function as a cell attachment factor and could play a key role in the adhesion of osteoclasts to the mineral matrix of bone .

CD-Length = 286 residues, only 12.6% aligned  
Score = 60.6 bits (146), Expect = 4e-11

Query: 5 ATEVFTPVVPTVD TYDGRGDSVVYGLRSKSKKFRRP 40  
Sbjct: 119 AATVFTPFVPTVD TNDGRGDSVAYGLRSKSKVFKVS 154

**Citing CD-Search:** Marchler-Bauer A, Bryant SH (2004), "CD-Search: protein domain annotations on the fly.", *Nucleic Acids Res.* 32:W327-331.

[Help](#) | [Disclaimer](#) | [Write to the Help Desk](#)  
[NCBI](#) | [NLM](#) | [NIH](#)



# Blast 2 Sequences results

PubMed

Entrez

BLAST

OMIM

Taxonomy

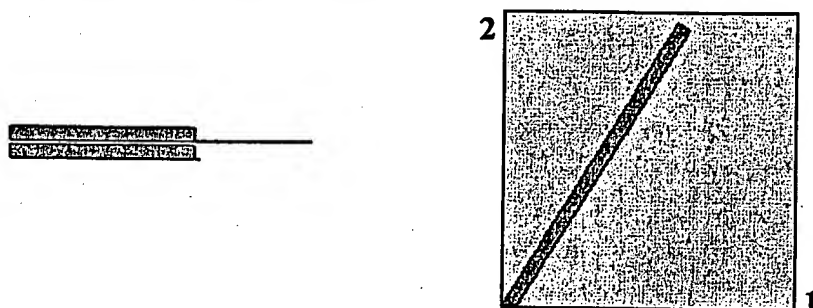
Structure

## BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.10 [Oct-19-2004]

Matrix: **BLOSUM62** gap open: **11** gap extension: **1**  
 x\_dropoff: **50** expect: **10.000** wordsize: **3** Filter ☒ Align

Sequence 1 cl|seq\_1 Length 63 (1 .. 63) SEQ ID NO: 11

Sequence 2 cl|seq\_2 Length 40 (1 .. 40) SEQ ID NO: 15



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 79.0 bits (193), Expect = 4e-14  
 Identities = 38/39 (97%), Positives = 38/39 (97%)

Query: 1 RSRRATEVFPTPVVPTVDTYDGRGDSVVYGRRSKSKKFRR 39  
 RSRRATEVFPTPVVPTVDTYDGRGDSVVYGRSKSKKFRR  
 Sbjct: 1 RSRRATEVFPTPVVPTVDTYDGRGDSVVYGLRSKSKKFRR 39

CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda K H  
 0.320 0.135 0.386

Gapped  
 Lambda K H  
 0.267 0.0410 0.140

Matrix: BLOSUM62

Gap Penalties: Existence: 11, Extension: 1

Number of Sequences: 1

Number of Hits to DB: 51

Number of extensions: 11

Number of successful extensions: 1

Number of sequences better than 10.0: 1

Number of HSP's better than 10.0 without gapping: 1

Number of HSP's gapped: 1

Number of HSP's successfully gapped: 1

Number of extra gapped extensions for HSPs above 10.0: 0

Length of query: 63

Length of database: 837,004,805  
Length adjustment: 38  
Effective length of query: 25  
Effective length of database: 837,004,767  
Effective search space: 20925119175  
Effective search space used: 20925119175  
Neighboring words threshold: 9  
Window for multiple hits: 0  
X1: 16 ( 7.4 bits)  
X2: 129 (49.7 bits)  
X3: 129 (49.7 bits)  
S1: 41 (21.8 bits)  
S2: 69 (31.2 bits)



## **Related Proceedings Appendix**

None

## **TABLE OF CONTENTS**

- (1) REAL PARTY IN INTEREST**
- (2) RELATED APPEALS AND INTERFERENCES**
- (3) STATUS OF CLAIMS ON APPEAL**
- (4) STATUS OF AMENDMENTS**
- (5) SUMMARY OF THE CLAIMED SUBJECT MATTER**
- (6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**
- (7) GROUPING OF CLAIMS**
- (8) ARGUMENTS**
  - (a) The Claimed Invention**
  - (b) Rejection Under 35 U.S.C. § 112, first paragraph**
- (9) SUMMARY AND CONCLUSION**

Claims Appendix: Claims On Appeal  
Evidence Appendix  
Related Proceedings Appendix  
Table of Contents